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Pulmonary immunomaturation in the neonatal horse: Age-related changes in the pulmonary cytokine milieu and circulating antigen-antibody complexes and their influence on macrophage phenotype and function

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Pulmonary immunomaturation in the neonatal horse: Age-related changes in the pulmonary cytokine milieu and circulating antigen-antibody complexes and their influence on macrophage phenotype and function

by

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

I would like to dedicate my dissertation to my late mother, Barbara T. Wiechert, RN, MS who encouraged my vision to pursue science and medicine. She reinforced my perseverant will and supported me through accomplishing my dreams. Her continued inspiration to never give up will remain a guiding force in my life.

TABLE OF CONTENTS

Page

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ABSTRACT

The prevalence of neonatal pneumonia exemplifies an age-related immune susceptibility among several mammalian species. The cause of this age-related susceptibility is primarily due to different mechanisms of immune defense within the neonate as compared to the adult. In horses, neonatal foals less than six months of age succumb to pneumonia caused by the intracellular, macrophage-tropic organism, *Rhodococcus equi*. Fulminant pneumonia in foals caused by *R. equi* develops as a slow progression of pyogranulomatous pneumonia and causes significant morbidity and mortality on equine breeding farms in horses aged two to six months.

Several studies within the literature provide explanations for this age related-susceptibility including a delayed production of a comprehensive array of endogenous antibody, altered cell-mediated immune responses, and diminished cytokine production by T cells; however, several gaps remain with respect to pulmonary specific immune changes within the ageing foal. The research reviewed in this dissertation focuses on the innate pulmonary cytokine milieu as well as the pulmonary alveolar macrophage (PAM) phenotype and function and how these immune environments and macrophage functions changed during the first year of life in a foal.

Bronchoalveolar lavage fluid (BALF) was collected from a cohort of foals during the first year of life and analyzed during a longitudinal study investigating the pulmonary cytokine milieu and the PAM phenotype and function. Increased concentrations of interleukin-4 (IL-4) within the BALF of neonatal foals early in life and consistent concentrations of tumor necrosis factor-alpha (TNF-α) within the first year of life were discovered. Interestingly, IL-4 concentrations increased within the first two months of

life and were virtually undetectable by six months of age. Furthermore, *ex vivo* PAM were discovered to demonstrate an increased susceptibility to *in vitro* intracellular infection with *R. equi* despite applied stimulus.

The final concept investigated, regarding the age-related susceptibility to pneumonia within neonatal horses, included measurement of naturally occurring circulating antigen-antibody complex concentrations. These immune complexes were studied as an alternative mechanism for macrophage activation ultimately resulting in enhanced intracellular microbicidal killing. Importantly, neonatal foals exhibited significantly lower concentrations of circulating immune complexes than adults providing additional evidence to support the notion that macrophages from foals are less capable of adopting a microbicidal phenotype.

CHAPTER 1. GENERAL INTRODUCTION

Statement of the Problem

Pneumonia is the single largest cause of death from infectious agents in children worldwide and accounts for 16% of all deaths of children under five years of age [1]. Despite the prevalence of pneumonia in children, adults suffer much less commonly from pneumonia. Even so, if adults develop pneumonia, the inciting factor is often related to concurrent sickness and hospitalization resulting in a community acquired pneumonia [2]. Still, less than 1% of adults under the age of 65 years develop community acquired pneumonia [2]. This well recognized age-related disparity of susceptibility to pneumonia is prevalent across all species. *Rhodococcus equi* is an important cause of foal pneumonia and is the leading cause of morbidity and mortality among foals aged one to six months [3]. While prevalent in foals aged less than 6 months, the disease is rare in adult horses, suggesting a difference in functional immunity [or a susceptibility]. The underlying causes for enhanced susceptibility in neonates and children are incompletely understood; however, it is known that their innate and adaptive immune responses are plastic and appear dependent on conditions of antigen and immunogen exposure.

Furthermore, the pulmonary immune response to antigen and immunogen is a complex interplay of events, and factors such as cytokines are well known for influencing macrophage phenotypes [4, 5]. Macrophage phenotype determines the extent of reactive oxygen species production [5]. Lack of production of reactive oxygen species (ROS) and nitrogen species (RONS) by alveolar macrophages has been documented in fulminant early-onset neonatal pneumonia [6], indicating a dampened response in fundamental lung defense mechanisms during this developmental period.

The macrophage phenotype and regulation of RONS are influenced by cytokines and chemokines indicating that the cytokine milieu of the phagocyte upon exposure to an antigen or immunogen is formative to the phagocyte's response. However, how the cytokine milieu of the neonatal vs. adult lungs contributes to production of RONS by tissue macrophages and pulmonary alveolar macrophages is not fully understood. Nonetheless, appreciation of the importance of innate macrophage responses to infection is garnering increased attention in the field of immunology; indeed, novel macrophage activation mechanisms that increase RONS production resulting in a consequential microbicidal effect are being explored and may hold promise for alternative antimicrobial strategies.

Interestingly, controversy exists as to whether foals have a bias for an anti-inflammatory or a pro-inflammatory immune response. For example, Wagner *et al.* investigated PBMC from neonates and found that they were unable to produce IL-4 following *in vitro* stimulation [7]. A conclusion was made that foal peripheral blood mononuclear cells (PBMC) were virtually incapable of producing IL-4, and foals demonstrated Th1- or pro-inflammatory- bias. Contrastingly, Breathnach *et al.* determined that foals were interferon-gamma deficient following collection and *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) and bronchoalveolar cells from 1, 3, and 6-month-old foals [8]. Breathnach *et al.* supported the notion that foals were generally Th2 biased while Wagner *et al.* concluded foals were not capable of Th2 responses [7, 8]. Thus, despite different context dependent studies that reached conflicting conclusions, these previous *in vitro* studies also represent experiments

whereby the methods effectively removed these cells from their local immune environment without first determining the cytokine exposure of those immune cells.

Thus, in addition to controversy regarding Th bias, or anti- vs. pro-inflammatory immune responses within foals, another major problem is the lack of site-specific immunomaturation information evaluating the local immune environment. To our knowledge, no cohort-based longitudinal studies have been performed that characterize pulmonary cytokine milieu changes from neonate to adult either within the equine or human pulmonary system. Furthermore, it therefore remains unclear how the cytokine milieu impacts the macrophage phenotype and resulting function. A better understanding of this process could enhance the knowledge regarding not only the predisposition of the young, but, also, how the immune system of the young vs. mature spatially and temporally differs from adults. This knowledge is expected to ultimately increase the future development of novel treatments for pneumonia in neonatal foals and, potentially, children.

Specific Aims

The overall goal of the studies performed in this dissertation is to better characterize the pulmonary immune environment and reveal qualitative and quantitative site-specific differences that may account for why the foal is more susceptible to developing pneumonia caused by *Rhodococcus equi* compared to the adult horse. The first specific aim is to determine the extent to which circulating and alveolar macrophages from neonatal foals and adult horses produce ROS and RONS. The working hypothesis for this aim is that macrophages from foals do not produce reactive oxygen and nitrogen intermediates (such as superoxide and peroxynitrite, respectively) at

levels similar to adult horses, which, in part, may account for their susceptibility to disease. The second specific aim is to determine the extent to which the cytokine milieu surrounding the macrophage affects macrophage phenotype and production of reactive oxygen and nitrogen intermediates. The working hypothesis for this aim is that macrophages from neonatal foals are exposed to a Type 2 cytokine milieu, namely IL-4, IL-13, IL-5, and IL-10, and consequently do not produce reactive oxygen and nitrogen intermediates at levels similar to adult horses. The third specific aim is to determine the extent to which circulating antigen-antibody complex concentrations differ in the foal vs. the adult horse in order to provide additional insight regarding the potential influence of these circulating antigen-antibody complexes on macrophage phenotype and function within the neonatal foal. The working hypothesis for this aim is that foals have lower concentrations of circulating antigen-antibody complexes due to decreased antigen exposure as compared to the adult and are therefore less able to shift their macrophage phenotype from a regulatory or tissue repair macrophage to a classically activated macrophage.

These hypotheses were tested by collecting peripheral blood and bronchoalveolar lavage fluid (BALF) from foals during the following ages of development: one week, one month, two months, 6 months, and 12 months of age in order to measure baseline cytokine concentrations as well as investigate the phenotype and function of peripheral and alveolar macrophages. The BALF was analyzed via ELISA for IL-4 and TNF-α for the aforementioned time points in foals (Chapter 2). Furthermore, the pulmonary cytokine milieu change in response to immunogen was investigated in the aging foal. Pulmonary alveolar macrophages (PAM) were isolated from BALF collected from

neonatal foals and adult horses and studied for production of ROS and RONS following *in vitro* stimulation in foals and adults (Chapter 3). Finally, serum from neonatal foals and adults was analyzed for circulating, soluble, antigen-antibody complexes and the concentrations were compared (Chapter 4).

Dissertation Organization

This dissertation describes the changes of the pulmonary cytokine milieu within the aging foal, comparing IL-4 and TNF-α BALF concentrations during the first year of the foal's life. Additionally, the foal's individual response to antigen or immunogen both within the *in vivo* pulmonary environment, and from isolated *ex vivo* macrophages stimulated *in vitro* was explored. The final concept investigated includes characterization of concentrations of circulating antigen-antibody complexes within neonatal foals as well as these antigen-antibody complexes' ability to induce production of ROS following macrophage stimulation. The dissertation is composed of five chapters and an appendix with the first chapter as the general introduction and literature review. Chapters 2, 3, and 4 are composed of the three individual manuscripts being prepared for the peer review journal submission, with the final chapter (Chapter 5) as conclusions and future directions.

The first and second papers (Chapters 2 and 3) are being submitted to the *Journal of Veterinary Immunology*. The third paper (Chapter 4) is being submitted to the *Journal of Equine Veterinary Education*. Finally, the appendix includes a modified version of a previously published journal article from the *Journal of Veterinary Clinical Pathology* in which age-related changes of the BALF cellular composition in neonatal foals and adult horses is characterized.

Literature Review

Scope

The scope of this literature review will focus on the developing immune system specifically within the foal and how increasing this knowledge can elucidate novel avenues for the prevention and treatment of disease, specifically pneumonia, for horses as well as other species, including humans. A background will be provided covering a general overview of *Rhodococcus equi* and its pathogenesis, as well as diagnosis and treatment of rhodococcal pneumonia in foals. The current knowledge regarding neonatal immunity will be covered with a focus on comparing the differences between the equine neonate and adult regarding the innate and adaptive immune responses. Finally, an explanation of the value of the equine lung as a model for pulmonary immunology studies will be explored.

Rhodococcus equi

Rhodococcus equi (*R. equi*) is an aerobic gram-positive, coccobacillus, facultative intracellular bacteria that lives in the soil, is found within fecal material from herbivores world-wide, and is an important pathogen among foals, farm animals, and humans [9-12]. Over the past three decades, *R. equi* has gained notoriety for infecting immunocompromised humans such as HIV/AIDS and transplant patients [13-16] and most recently for recurrent meningitis following pneumonia in a human patient [17].

This multi-host pathogen is also known to cause diseases in a variety of other domestic animals. *R. equi* can be isolated from 3-5% of apparently healthy pigs usually from their submandibular lymph nodes [18]. *R. equi* has also caused disease in swine that usually are presented with granulomatous lymphadenitis [18-21]. However,

experimental infection studies have failed to reproduce granulomatous lymphadenitis in pigs [19-22].

An abattoir survey in Ireland between 1997-98 found 4% of tuberculosis-like granulomas present in the retropharyngeal, bronchial and mediastinal lymph nodes from cattle contained *R. equi* identified by culture [18, 23]. Bronchopneumonia, mastitis, metritis, ulcerative lymphangitis, and septic arthritis have also been diseases in cattle caused by *R. equi* [12, 18]. In smaller domestic species, such as the cat and dog, *R. equi* has been a documented cause of several diseases ranging from pneumonia, wound infections, subcutaneous abscesses, vaginitis, and hepatitis, to osteomyelitis, myositis, and joint infections [24].

Three species adapted virulence plasmids known as VapA, VapB, and VapN are currently recognized for *R. equi* and each type can be isolated from the equine, bovine, and porcine species, respectively [25]. However, any of these plasmids can be isolated from humans infected with *R. equi* suggesting a potential for zoonosis [25-26]. A pathogenicity island is located within each of these plasmids which are acquired by horizontal gene transfer [25, 26]. These plasmid types differ in genetic composition; however, all contain a novel family of genes known as the virulence associated proteins or vap family [25-27].

Previous experimental studies have demonstrated that the major virulence determinant, VapA, which is encoded on the pVAPA-type plasmid, is crucial for bacterial replication within macrophages and acts through disrupting normal development of the phagosome [18, 25, 28]. Specifically, once phagocytized, VapA enables avoidance of intracellular killing by the host through disrupting the events proceeding the phago-

lysosome fusion and lysosome reformation allowing replication of *R. equi* to cause a pyogranulomatous abscess [29]

Due to its virulence mechanism, *R. equi* is most recognized for the cause of severe pyogranulomatous pneumonia in foals and poses a significant economic burden on the equine breeding industry [18, 30].

Diagnosis and treatment of pneumonia caused by *Rhodococcus equi*

Due to the slow spread of this granulomatous disease and the foal's ability to compensate during the primary stages of the disease, early diagnosis of pneumonia caused by *R. equi* can be challenging. When pulmonary disease is apparent, foals are presented with the following clinical signs: nasal discharge, cough, tachypnea, fever, and lethargy. Foals may also be presented with other extrapulmonary disorders; however, for the scope of this literature review, diagnosis and treatment of pneumonia will remain the focus.

In addition to pneumonia, extrapulmonary lesions caused by *R. equi* are also common. Intestinal lesions caused by *R. equi* are characterized by multifocal ulcerative enterocolitis and typhlitis over the area of Peyer's-patches with granulomatous or suppurative inflammation of the mesenteric and or colonic lymph nodes and foals afflicted with abdominal abscesses have a poor prognosis [18, 31]. A study reported that up to 50% of foals with *R. equi* have intestinal lesions that are found during necropsy [18, 31, 32].

Furthermore, bacteremic spread of the organism from the lungs or gastrointestinal (GI) tract can result in septic arthritis or more commonly osteomyelitis [18, 31]. Occasionally, foals develop septic arthritis or osteomyelitis without apparent lung

involvement. Another extrapulmonary disorder that occurs in foals with *R. equi* is immune mediated inflammation within the joints and is present in approximately one-quarter to one-third of all affected foals [18, 31, 33].

Taking all extrapulmonary disorders into consideration, a study examined 150 foals with *R. equi* that were admitted to a teaching hospital [34]. At least 1 of 39 extrapulmonary diseases was identified in 74% of cases and survival was significantly lower among foals with extrapulmonary disease: 43% compared to 82% in foals only with pneumonia [18, 34].

An accurate diagnosis depends on the signalment and history of the foal. Foals are usually presented between 3 weeks and 5 months of age and the farm from which they are presented often has a history of previous cases of pneumonia caused by *R. equi* [31]. A thorough physical examination should be conducted. Bronchovesicular sounds can be appreciated during lung auscultation and these are commonly referred to as 'rattles'. A complete blood count should be performed and often will reveal a leukocytosis, neutrophilia and hyperfibrinogenemia. Thoracic radiographs and ultrasound can take place to confirm the presence of granulomatous lesions within the pulmonary parenchyma. A trans-tracheal wash can be executed for definitive culture of *R. equi* [18, 31].

Treatment of the organism includes combination antibiotic therapy including a macrolide and rifampin. Clarithromycin is the preferred macrolide as it is lipophilic and is found to have a greater concentration within the pulmonary epithelial lining fluid and the alveolus [35]. Supportive care may include nasal oxygen insufflation, intravenous fluid therapy, hyperimmune plasma and anti-inflammatory therapy. Depending on the

severity of the disease, treatment can extend from three weeks up to 12 weeks and beyond.

Breeding farms with a known history of rhodococcal pneumonia have adopted screening methods in order to identify which subclinical foals should be treated with antibiotics [36]. Breeding farms have used ultrasound as a screening tool for subclinical disease cut-offs, including either a sum of all lesions or an abscess score. In one scoring system, a score of 1-10 cm or less and the foal lesion scores were considered below the cut-off. Thus, treatment was deemed unnecessary as these lesions were considered likely to resolve on their own; however, no controlled studies were performed evaluating this method. Antimicrobial resistance of *R. equi* is of great concern and these various screening methods adopted across breeding farms have been implicated as a possible potentiating mechanism of resistance to antibiotics. Two independent studies in 2012 showed that 80-90% of foals with subclinical disease on endemic farms cleared the infection without treatment [37, 38]. However, the problem remains that there is no way to ascertain which foals with subclinical disease will progress and develop clinical disease requiring treatment.

Reported in 2017, resistance to macrolides and rifampin in isolates of *R. equi* cultured from horses is increasing, with isolates resistant to all macrolides and rifampin now being cultured from up to 40% of infected foals at several farms [39]. This trend makes increasing knowledge, regarding the immunologic susceptibility of foals, paramount to uncovering potential, new avenues of alternative treatments.

Neonatal immunity

While pneumonia caused by *R. equi* is prevalent in foals aged less than 6 months, the disease is exceedingly rare in adults, suggesting a deficit in functional immunity. As an intracellular macrophage-tropic organism, *R. equi* has a pathogenesis similar to *Mycobacterium tuberculosis* and is able to evade the host's immune system within neonates and immunocompromised patients [18, 29, 31, 40]. The immune response against intracellular macrophage-tropic organisms is a complex interplay of events involving both the innate and adaptive immune response working in concert to protect the host. Major differences have been identified among neonates and adults.

As the innate immune system is the first line of defense against pathogens, it also greatly influences the adaptive immune response. Microbial organisms are recognized through a set of molecular pattern recognition receptors present on the extracellular or intracellular membranes of many cells. These bacterial and viral patterns are recognized through a family of receptors known as the Toll-like-receptors and C-type lectins. Once these receptors recognize a pattern, they signal through various downstream cellular mechanisms and this signaling often results in the production of cytokines and chemokines which influence the surrounding cellular milieu and lay the foundation for the subsequent immunologic events.

Effects of cytokines can either be from the cytokine or the degree of the cytokine's receptor expression profile. Nonetheless, if recognized, cytokines are well known for influencing macrophage phenotypes. Regarding rhodococcal pneumonia, determining the baseline pulmonary cytokine milieu within the neonatal foal would be expected to reveal potential reasons why foals are susceptible to developing pneumonia from *R. equi* [4, 5, 41]. The current areas of research that need further investigation,

which are applicable to these studies, include age-related and site-specific information regarding the pulmonary cytokine milieu, and changes vis-à-vis macrophage immunophenotypic responses and activation.

Macrophage activation status directly correlates with resolution of *R. equi* disease. Lack of production of nitric oxide and peroxynitrite by alveolar macrophages has been documented in fulminant early-onset neonatal pneumonia [6], indicating an age-related deficit in fundamental defense mechanisms during this developmental period. Indeed, two of the most important effector systems of macrophages that are paramount to innate immune responses include the NADPH oxidase (also known as phox) and inducible nitric oxide synthase (iNOS) pathways which account for the generation of superoxide (O2– •) and nitric oxide (NO•) radicals, respectively.

Together, superoxide and nitric oxide can form the highly reactive molecule, peroxynitrite [42, 43]. These three reactive intermediates have broad antimicrobial effects and their use as effector molecules is conserved across the plant and animal kingdoms [44-46].

Concerning the general categorization of macrophages and their production of reactive intermediates, classically activated macrophages are efficient at producing free radicals and thereby are effective killers of intracellular pathogens while regulatory and tissue repair macrophages are inefficient killers [4, 5]. In recent years, experiments investigating the effect of cytokine priming on macrophage function following *in vitro* infections with *R. equi* have been studied using adult equine macrophages. Of note, pro-inflammatory cytokines like tumor-necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) were found to improve intracellular killing of *R. equi* [47]. Additionally,

macrophages of different lineages and from different ages of foals were harvested and infected, *ex vivo*, with *R. equi*. According to Berghaus *et al.*, regardless of the macrophage lineage, foal macrophages at three months of age permitted higher replication of intracellular *R. equi* and, overall, alveolar macrophages were more susceptible to a higher bacterial load [48].

Given the direct impact that cytokines have on macrophages, these professional phagocytes also produce cytokines in response to not only their immediate environment, but in response to pathogen recognition. The cytokines that macrophages produce can be used to characterize their phenotype, as well. Consistent with current data, our previous work indicated that stimulated monocyte-derived macrophages (MDM) from neonates produce more IL-10 than similarly derived macrophages from adults, suggesting a fundamental difference in cytokine production between neonates and adults [49].

Antigen presenting cells, like the macrophage, represent the bridge between the innate and the adaptive immune system as they present processed antigen to T cells and B cells through their MHC receptors, which subsequently become activated, provided the appropriate signals are present, and orchestrate the immediate antibody and/or cell-mediated response.

Concerning the adaptive immune response, variations between the neonatal and adult immune system have been documented [50]. Importantly, Hostetter *et al.* demonstrated a significantly decreased lymphocyte population within the BALF of foals compared to adults [51]. The key players of the adaptive immune response are lymphocytes which can be divided into several categories including T-helper cells, cytotoxic T cells, and B cells. Of the T cell population, CD4+ and CD8+ cells, in

particular, are important regarding initiation and maintenance of cell-mediated immunity, T cell mediated cytotoxicity, and B cell expansion, proliferation and antibody isotype class-switching as well as the formation of a memory response [49]. As such, lymphocytes have been extensively studied from aging foals [7, 8, 53-56].

Interestingly, CD4+ and CD8+ cells occur in significantly fewer numbers in neonatal horses than adult horses and were found to increase linearly in foals up to 3 months of age [54]. This delay in CD4+Th cell development poses a risk for neonatal horses especially concerning intracellular pathogens.

IFN-γ is an important cytokine causing antimicrobial activity against intracellular pathogens, like *R. equi*. IFN-γ not only activates the adaptive immune system while promoting the development of high-affinity antigen-specific T and B cell responses and immunological memory but also directly acts on neighboring endothelial and epithelial cells to promote an antimicrobial state [58]. Decreased IFN-γ production from Th1 cells and cytotoxic T cells has been observed shortly after birth and only gradually increases over the first year of life [8, 31, 59]. Given this ineffective IFN-γ production in foals, a great disadvantage prevails for successful resolution of pneumonia caused by *R. equi*.

Regarding the humoral portion of the adaptive immune response, foals rely heavily on maternal antibody as their own humoral system begins to slowly produce endogenous antibody. Maternal immunity greatly impacts the immune system of the developing fetus and the acquisition of passive immunity is solely influenced by the maternal-fetal placenta. Equine fetal growth occurs through an epitheliochorial placental maternal-fetal unit which impedes the transfer of maternal antibody (mtAb). Thus, foals are born with a paucity of circulating maternal antibody until they ingest colostrum from

the mare. This period between birth and colostrum ingestion is critical and imposes great potential for the foal to develop sepsis if passive transfer of immunity is not achieved. Within three hours after birth, the foal should ingest colostrum in order to remain protected against infectious diseases [60]. The ingested mtAb protects the foal against several infectious diseases during the diminution of colostral antibody and the simultaneous increase in endogenous antibody. Additional factors including T cells and cytokines are adsorbed along with mtAb and are thought to influence the foal's immune system [59].

Concerning endogenous production of the seven different immunoglobulin-g isotypes within the foal, a few isotypes undergo early development before birth and mature within the foal by 3 months of life and include IgG1, IgG3, IgG5 as well as immunoglobulin-A (IgA). However, IgG4, IgG7 as well as immunoglobulin-E (IgE) develop at a slower rate during the first year of life [59]. Each antibody isotype varies in its effector function although a few have an overlapping effector function. According to Lewis *et al.* 2008, IgG1, IgG3, IgG4, IgG5 and IgG7 have the ability, once complexed with antigen, to elicit a strong respiratory burst from macrophages and are presumed to have great affinity to Fc receptors [61]. Regarding complement activation, immune complexes of IgG1, IgG3, IgG4 and IgG7 all bind C1Q and activate the classical complement pathway [61]. IgG2 and IgG6 did not induce a respiratory burst or bind complement and were implied as undesirable isotypes to produce during vaccination, although this is context dependent and can change depending on the immune environment [58]. Concerning any antibody isotype, foals have been reported to have

attenuated responses to vaccination prior to six months of age due to interference of mtAb as well as inefficient Th2 responses [59, 62].

Collectively, these studies demonstrate that foals have decreased Th1 and Th2 responses, both pro- and anti-inflammatory immune responses, decreased numbers of circulating CD4+ and CD8+ T cells during the first few months of life, fewer lymphocytes and higher numbers of macrophages within BALF (Figure 1-1) and reduced responses to vaccines. The greatest impact that manifests as a consequence of these aforementioned age-related immunologic differences between foals and adults is perhaps foal pneumonia caused by *Rhodococcus equi*. Thus, experiments that increase the information regarding site-specific immunologic knowledge as an aid to explain the discrepancies documented within the literature are not only critical for the field but would potentially uncover treatment options for immunomodulation of foal pneumonia caused by the intracellular pathogen, *R. equi*.

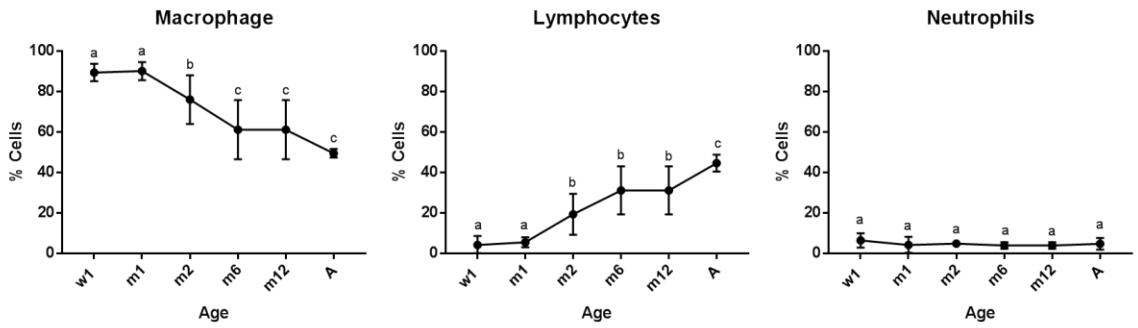

Figure 1-1. Cell populations within bronchoalveolar lavage fluid (BALF) from young foals are dominant in macrophages. Bronchoalveolar lavage fluid samples from foals, during their first year of life, were submitted to the Clinical Pathology department at Iowa State University, College of Veterinary Medicine for analysis. A cytospin was generated and a 300-cell count differential was performed. Cell populations are shown as a percentage of total cells within the differential. Macrophages decreased over time ($p < 0.0001$) and lymphocytes increased over time ($p < 0.0001$). No significant changes in neutrophils or any other cell type were appreciated. By twelve months of age, foals had similar populations of cells compared to their adult counterparts.

Equine model for pulmonary studies

Pneumonia caused by *R. equi* naturally occurs only within foals and not adults, making the horse an ideal species to study neonatal pulmonary immunologic responses in order to reveal critical differences between the neonate and the adult immune system. Moreover, given the intraspecies parallel between susceptibility to development of pneumonia caused by *R. equi* in both foals and immunocompromised humans, the equine model provides many advantages when seeking to understand developing pulmonary immunomaturation.

The equine neonatal model has several inherent strengths in that pulmonary alveolar and monocyte-derived macrophages may be repeatedly obtained with relative ease and in sufficient quantities to carry out immunologic studies. The pulmonary anatomy of the horse is more similar to the human than the murine model for several

reasons. These similarities are demonstrated both grossly as well as ultra-structurally [63-66].

First, the lobular and branching pattern within the horse more closely resembles the human than the mouse. Anatomical terminology may differ among various researchers and in order to develop consistent terminology system, Nakakuki, a comparative anatomist, studied various mammal lungs and developed a consistent terminology making comparison between mammals unified. As such, the equine lung is divided into the cranial and caudal lobes via the cardiac notch on each side. Within the right lung, the accessory lobe is also present. Concerning the bronchial branching patterns, the equine lung is divided into four bilateral sections: the cranial, middle, caudal and accessory lobes [63]. When examining the bilateral bronchiole organization on either side, the bronchioles are divided into the dorsal, lateral, ventral and medial sections [63].

Furthermore, on the dorsal aspect of the lung, the first bronchiole from the dorsal bronchiole system forms the cranial lobe [63]. On the lateral aspect, the middle lobe is formed by the first bronchiole of the lateral bronchiole system. The accessory lobe originates from the first bronchiole of the ventral bronchiole system. Finally, the remaining bronchioles of the dorsal, lateral and ventral bronchiole systems and all bronchioles of the medial bronchiole system constitute the caudal lobe [63]. The equine branching pattern resembles the human branching pattern closely; and both are known as an irregular dichotomous or trichotomous bronchiole branching pattern while mice display a primarily monopodial branching pattern [66, 69].

Concerning the histopathologic features of the pulmonary associated lymphoid tissue, Mair *et al.* studied the distribution of mucosa-associated lymphoid tissue along the equine respiratory tract using light microscopy to evaluate histologic sections [67]. This study determined that extending from the nasal vestibule to bronchioles, measuring 2 - 4 mm in diameter, intra-epithelial lymphocytes were present in the lamina propria with isolated lymphoid patches and occasional nodules were common in the bronchioles, referred to as bronchiole-associated-lymphoid-tissue (BRALT). However, the density of this pattern was highly variable among the 10 horses of various breeds ranging in age from 2 to 12 years that were surveyed [67]. Bronchus-associated lymphoid tissue (BALT) was infrequently encountered while nasal-associated lymphoid tissue (NALT) nodules were dispersed throughout the nasal cavity, nasopharynx and adjacent to the auditory tube within the upper respiratory tract. Additionally, laryngeal- and tracheal-associated lymphoid tissue (LTALT) was also identified, but this was limited to the epiglottis, arytenoid and rostra1 trachea. Within these locations, discrete lymphoid masses were comprised of primary or secondary nodules, a parafollicular zone and a dome area. Above these discrete lymphoid masses, Nakakuki documented a lympho-epithelium with infolding patterns that potentially functioned to enhance antigen-trapping [63].

The presence of BALT in horses and humans is also similar although there is much variation between individuals, and the presence of BALT in humans has been regarded as controversial. However, Kawamata infers that BALT in humans is involved in the initiation of bronchopulmonary immune responses to inhaled antigens and pathogens [65]. This conclusion was also reached regarding horses in a study

investigating the histological features of the equine pulmonary system [67]. The equine pulmonary system contains a complex and dynamic local lymphoid system extending from the nasal cavity to the bronchioles and is most likely the result of stimulation from particulate matter and microbial organisms [68]. Ultimately, unlike the rodent model [66, 69], the gross and microscopic anatomy and presumed function make the horse an ideal model to study respiratory disease affecting neonates and young organisms as a result of immunologic differences.

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CHAPTER 2. AGE-RELATED CHANGES IN THE PULMONARY CYTOKINE MILIEU OF EQUINE BRONCHOALVEOLAR LAVAGE FLUID

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Abstract

Many intracellular pathogens, like *Rhodococcus equi* , preferentially cause pneumonia in the young and immunocompromised across species. Specifically, foals less than six months of age suffer from pneumonia caused by *Rhodococcus equi* while adult horses do not succumb to this disease. This naturally occurring disease model highlights a critical immunologic difference between neonatal and young species vs. their adult counterparts. Although many advances have been made exploring the adaptive immune response within neonatal horses, none have investigated the innate pulmonary cytokine milieu and how this milieu changes as the foal ages as well as in response to immunogen. Cytokines affect several cellular processes and can either up-regulate or down-regulate inflammation. Related to the pulmonary immune environment, pro-inflammatory cytokines impact macrophage activation status and can influence their microbicidal activity against macrophage-tropic organisms like *R. equi*. Our central hypothesis is that the cytokine milieu within the BALF is biased toward an anti-

inflammatory or regulatory milieu early in a foal's life. Specifically, we hypothesized that foals would have increased concentrations of interleukin-4 (IL-4) and lower concentrations of tumor necrosis factor-alpha (TNF-α). To test our hypotheses, BALF was sampled from a cohort of foals at week 1, 4, 8, 26, and 52 weeks of age and analyzed to determine the cytokine milieu concentration across the first year of life.

Introduction

Pneumonia remains the number one killer of young children globally and is responsible for an estimated mortality rate between16 and 18% of children under 5 years of age [1, 2]. An estimated 120 million new cases of pneumonia in children occur globally each year [1]. In contrast, pneumonia in adults is significantly less common [3]. This suggests an underlying susceptibility of the immune system in neonatal and young mammals. In particular, foals suffer from pneumonia caused by *Rhodococcus equi* whereas adult horses do not acquire pneumonia caused by *R. equi*. The bacterial species, *R. equi*, co-evolved in concert with the equine species and its ability to cause pneumonia in neonatal foals highlights a unique infectious disease development process only observed within foals less than six months of age. While neonates have an increased susceptibility to multiple pathogens, there is a dynamic immune process occurring between *R. equi* and neonatal foals resulting in pneumonia but only in the young host. This represents a naturally occurring disease model and provides an exceptional opportunity to understand intracellular pathogens in the context of a neonatal immune system.

Classically activated macrophages are potent killers of intracellular microorganisms by their upregulation of reactive intermediates although other

phenotypes of macrophages, that are less able to kill intracellular organisms, exist as well [4]. Macrophage activation status depends on their immediate immune environment [4]. *R. equi* is an intracellular macrophage-tropic organism that evades the macrophage phagolysosome through its virulence plasmid, VapA [5]. Given that infection with *R. equi* is acquired through inhalation and preferentially infects pulmonary alveolar macrophages causing pneumonia, understanding the immediate immune environment of the lung within the neonate is imperative in order to determine its implicit impact upon the macrophage.

Previous work has been performed investigating the expression of the cytokine-specific mRNA profile from bronchoalveolar lavage fluid (BALF) cells as well as from peripheral blood mononuclear cells (PBMC) from foals. To date, there is a lack of information regarding a longitudinal characterization of the extracellular cytokine milieu in neonatal foals specifically within the lung. The aim of this study was to characterize the pulmonary cytokine milieu by measuring the concentration of a pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α), as well as an anti-inflammatory/regulatory cytokine, interleukin-4 (IL-4), present in the BALF. Samples were acquired from a cohort of neonatal foals beginning during the first week of life and were collected until one year of age.

Determining the balance of cytokines present within the pulmonary BALF from foals was expected to provide insight regarding the relative exposure of pulmonary alveolar macrophages (PAM) to either a pro-inflammatory or anti-inflammatory environment. The central hypothesis of this work is that the neonatal pulmonary cytokine milieu is biased toward an anti-inflammatory or regulatory milieu. To test this hypothesis,

BALF was acquired from 12 foals at the following time points: 1 week, 4 weeks, 8 weeks, 26 weeks, and 52 weeks of age. At each time point, on day 0 the left lobe of the lung was used as the control side and BALF was collected from the left side first. Sterile PBS was instilled into the left lobe. Subsequently, a baseline BALF sample from the right lung was acquired from the right lobe of the lung. Then, heat-killed *R. equi* was instilled into the right side of the lung. From each side of the lung, BALF was then collected on day 1 and day 4 after heat-killed *R. equi* instillation in order to study the response to an immunogen within the neonatal lung. Heat-killed *R. equi* was instilled into the lung to determine if the foals would exhibit a pro-inflammatory response over time reflecting a progressive decrease in susceptibility to pneumonia as foals age.

Materials and Methods

Experimental Design

In this longitudinal study, BALF sampling started during the neonatal period of the foal and extended through to sexual maturity as immunocompetency of the lung was acquired. The equine model affords collection of ample material from a neonate, the opportunity to prime a selected bronchus with an immunogen and collect bronchoalveolar lavage samples from the same site at repeated intervals, a contralateral bronchus for intra-animal saline control, and a time to maturity that accommodates studies of immunomaturation. Moreover, this equine model takes advantage of: (1) *R. equi*, a respiratory pathogen of horses known to infect neonates but that is effectively cleared by the adult, (2) our recent contribution documenting an anti-inflammatory/regulatory cytokine bias to equine neonatal macrophage responses [6], and (3) the pulmonary

anatomical and immunologic similarities between the horse and human providing a potential platform for translational neonatal immunologic results.

Animals

Twelve foals, of various breeds (Thoroughbreds and Quarter Horses), were used for this study which was approved by the Iowa State University Animal Care and Use Committee. The foals were born at the Iowa State Research Barn in Ames, IA. Within the first 48 hours of life, a physical exam, CBC, serum biochemistry, status of passive transfer of antibody and a complete history were assessed to assure healthy subjects. This age of foal was selected as (1) it is most likely to demonstrate functional differences when compared with the adult; (2) the rate of functional maturation of equine macrophages is unknown; and (3) infection with *R. equi* is posited to occur within the first two weeks of life [7]. Due to attrition of the subjects, an incomplete data set was collected and analyzed.

Foals were housed with their respective dams in a separate facility from the rest of the herd. All foals were weaned from their dam around four months of age. All dams and weaned foals were kept on a dry lot and were fed a mix of alfalfa and grass hay with supplemental complete pelleted feed.

Immunogen Preparation

Rhodococcus equi , strain T194, was provided as a clinical isolate from a pneumonic foal by Dr. Ronald Griffith, Iowa State University. A single colony was seeded to 20 mL of brain, heart infusion (BHI, BD, Franklin Lakes, NJ) media at a starting optical density of 0.12. The culture was incubated at 37° C for approximately

five hours until the O.D. reached 0.25, which corresponded to 10^8 bacteria per mL. Bacteria were washed in sterile PBS and resuspended in phagocytosis buffer. *R. equi* were diluted to 75 µg/mL in PBS in a 1 mL microcentrifuge tube and then placed in a heat block for 90 minutes and held at 75° C for 90 minutes to kill the bacteria. To confirm killing occurred, a sample was streaked onto a BHI plate at 37° C, 5% CO_2 and incubated for 48-72 hours to confirm that no growth occurred following the heat-kill procedure. The killed bacteria were then further diluted to 1 µg/mL. A total of 5 µg of the heat killed *R. equi* diluted in sterile PBS was used each time during pulmonary immunogen instillation to the right side of the lung.

Bronchoalveolar Lavage

Bronchoalveolar lavage fluid (BALF) was obtained from the neonatal period through adulthood. BAL were performed on day zero (d0), day one (d1), and day four (d4) beginning at week one (w1), week four (w4), week eight (w8), week 26 (w26) and week 52 (w52) of age. BALF was always collected from the left lung first; then PBS was instilled as the control. The endoscope was then moved to the right side of the lung, for BALF collection. Prior to removing the endoscope from the right side of the lung immunogen was instilled (5 µg of heat-killed *R. equi*) on day 0 after each week time point. The BAL was performed as previously described [8]. The lavage was performed using an endoscope appropriate to age and size of the animal with 60 mL syringes containing sterile saline. After insertion of the endoscope into the elected site, BALF was harvested in syringes by aspiration of the instilled saline.

A small amount of clarified BALF was aliquoted and frozen at -20° C for ELISA cytokine analysis and an EDTA tube containing 1 mL of BALF was sent for cell count

and cytology as in Hostetter *et al.*, 2017 (Appendix) [9]. Remaining fluid was placed into 50 mL conical tubes and centrifuged at 500 x *g* for 10 minutes at 4° C. Pellets were washed using DPBS (Mediatech, CellGro, Inc., Manassas, VA) containing decreasing amounts antibiotic/antimycotic (Lonza, Basel Switzerland) (wash 1: 2.5 µg amphotericin-b/ml; wash 2: 0.1 µg amphotericin-b/mL, 2 U of penicillin/ml, and 2 U streptomycin/ml). Cells were pelleted after the second wash and resuspended in freezing medium containing 90% fetal bovine serum (FBS) (Atlanta Biologicals Inc., Flowery Branch, FL) and 10% DMSO (Sigma-Aldrich, St. Louis, MO). Cells were placed in an isopropanol Mr. Frosty® (Nalgene, Rochester, NY) freezing container at -80° C for 24-72 hours prior to transfer to liquid nitrogen.

Cytokine Measurement

To characterize the pulmonary cytokine environment, ELISAs were performed on BALF. BALF was analyzed for equine IL-4 and TNF-α using R&D Duo-Set ELISA Kits (R&D Systems, Minneapolis, MN). The assay standard concentration range for both IL-4 and TNF- α was 31.3 pg/mL - 2,000 pg/mL. Samples were run neat (BALF as collected), and in triplicates. Absorbance was measured using FLUOstar® Omega (BMG Labtech, Cary, NC) fluorimeter at 425 nm. Optical density values were converted to pg/mL using a 4-parameter fit function using the Omega software. Ratios of TNF-α:IL-4 were created for cytokine normalization of data.

Statistical Analysis

Data were analyzed with the statistical software package SAS (version 9.4, SAS Institute Inc., Cary, NC). The concentration of cytokines and the ratio of the

concentration of cytokines in the BAL were log_{10}-transformed prior to statistical analysis to ensure normally distributed residuals. Data were analyzed by using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) as a split-plot -in- time design with repeated measures with foal as the whole-plot and foal age as the sub-plot. "Foal" was considered random and "day relative to heat-killed *R. equi* exposure" was a repeated measure. To calculate changes in cytokine concentration relative to day 0, the difference between the cytokine concentration and the concentration on day 0 was found for both days 1 and 4. A constant was added to each value to eliminate negative numbers prior to log10-transformation. After statistical comparisons were made, the values were back-transformed and the constant was subtracted from the back-transformed data. All data presented are LSMeans and SEM following back-transformation. Mean separations were protected by an overall F-test and differences were considered significant when $p < 0.05$. Data and significant values for the TNCC and cellular proportions is being reported from Hostetter *et al.* 2017 (Appendix) [9].

Results

BALF Cell Proportions and Total Nucleated Cell Count (TNCC) Changed as Foals Age

The cellular proportions of the BALF fluid analyzed from foals significantly changed across the first year of age. Foals less than eight weeks of age had the highest proportion of macrophages compared to 26-week-old and 52-week-old horses (Figure 2-1). Interestingly, foals aged 26 weeks and greater had higher proportions of lymphocytes when compared to younger foals (Figure 2-1).

Foals Had Increased IL-4 Concentrations within BALF Early in Life While BALF TNF-α Concentrations Did Not Change During the First Year of Life

Foals exhibited higher concentrations of IL-4 in the BALF at time points ≤ six months of age compared to that measured at one year of life (Figure 2-2). Furthermore, the baseline concentration of IL-4 increased from one week to four weeks of age and either exhibited similar concentrations or a slightly higher concentration by eight weeks of age. Around 26 weeks (six months of age), the concentration of IL-4 significantly decreased and was also low at one year of age (Figure 2-2). Foals had significantly greater IL-4 concentrations during week one compared to six months of age ($p < 0.0015$) (Figure 2-2). At four weeks of age, the BALF IL-4 concentration was significantly greater than weeks 26 and 52 ($p < 0.0005$) ($p < 0.025$, respectively) (Figure 2-2). By eight weeks of age, the BALF IL-4 concentration of foals was significantly greater than six months of age ($p < 0.003$) (Figure 2-2) and was significantly less than 52 weeks of age within the left side of the lung ($p < 0.0467$) (Figure 2-2B). Furthermore, this pattern was largely consistent for both the right and left sides of the lungs, which were sampled, processed, and analyzed separately. In contrast, BALF concentrations of TNF-α did not significantly change as the foal aged and this consistency was present in both the right and left lungs ($p > 0.05$; Figure 2-3).

The Foal Ratio of IL-4:TNF-α Significantly Changed During the First Year of Life

In order to normalize the cytokine concentrations and account for any minor changes of volume differences between lung sides during the BAL procedure, the mean of the right and left cytokine values on day 0 for each foal respective of the age of the regulatory/tissue repair cytokine, IL-4 was divided by the mean of the pro-inflammatory

cytokine, TNF-α, to determine changes relative to each other. The ratio significantly changed ($p < 0.05$) (Figure 2-4) as the foals aged but the difference was primarily driven by changes within IL-4 BALF concentrations (Figure 2-2) as there were no statistically significant differences in BALF TNF-α concentrations as the foals aged (Figure 2-3). Early in life, the ratio was a lower number, and this was due to the relatively higher concentration of IL-4 compared to the lower IL-4 concentration measured later in life (Figure 2-4). By six months of age, the ratio markedly increased, and this is due to a dramatic decrease in IL-4 concentration (Figure 2-2, Figure 2-4).

The Pattern of Cytokine Response in Foals to Pulmonary Instillation of Heat-Killed *R. equi* vs. PBS Changed During the First Year of Life

In order to determine if there were immune response differences in the local pulmonary cytokine profile in neonatal foals during the first year of their life, immunogen (heat-killed *R. equi* (5 μg)) was instilled into the right lung and PBS was instilled into the left lung. BALF was collected one day and four days post-instillation. During early life (less than two months of age) within the right lung, BALF IL-4 significantly increased in response to heat-killed *R. equi* during w1 and w8 of life ($p < 0.05$) (Figure 2-5).

Of note, BALF IL-4 concentrations were observed at their highest concentration during week four (Figure 2-2) yet, the BALF concentration of IL-4 decreased in response to *R. equi* which may suggest either the maximal pulmonary secretion level of cytokines or a sensitivity limit of the ELISA detection range. Following week eight, virtually no differences regarding response to antigen were observed. Concerning the control lung (left lung), no significant three-way interaction between age, week, and day occurred (Figure 2-5) ($p > 0.05$). There were minor changes in the BALF IL-4 concentration on

the left side of the lung during day 1 and day 4 post-instillation; however, the magnitude of these changes was less when compared to the right side and no significant difference was observed. According to the F-test, there was no significant interaction between day and response to *R. equi* within the right lung BALF (data not shown) for TNF-α concentrations.

Discussion

This study compared the cytokine milieu, specifically IL-4 and TNF-α, within the BALF collected from a cohort of healthy foals. Additionally, this is the first study to assess age-related changes of the pulmonary cytokine milieu within the same cohort of foals during their first year of life. Our results indicate that foals contain higher concentrations of baseline BALF IL-4 concentrations earlier in life. Additionally, the local pulmonary cellular population responds differently to instilled antigen earlier in life by producing increased concentrations of IL-4. Interestingly, the concentrations of TNF-α do not change as a result of age. These data indicate that the pulmonary immune environment of the foal may be either biased toward an anti-inflammatory or regulatory immune environment which may impact the surrounding pulmonary alveolar macrophage population by influencing their phenotype to adopt an anti-inflammatory and tissue repair/regulatory phenotype. Therefore, as a microbicidal phenotype is not elicited, efficient intracellular-killing of macrophage-tropic organisms does not occur. Overall, these results reveal a possible cause for an increased susceptibility to pneumonia caused by *R. equi* within foals.

Interleukin-4 is a primary Th2 regulatory, and tissue repair cytokine that is produced by a variety of immune cells including lymphocytes, basophils, and eosinophils

and is most regarded for its immune physiologic role during parasitic infections, antibody production and class switching of B-cells following vaccination, as well as its aberrant role during allergic diseases [10]. As an equally important function, though less promulgated, IL-4 also serves as an important factor in maintaining metabolic homeostasis and interest in this area of research is increasing [11]. Production of IL-4 is not limited to immune cells and can also be produced by a variety of others including bronchial epithelial cells [12, 13]. Interestingly, a study demonstrating that human bronchial epithelial cells produce IL-4 suggested that IL-4 set up an anti-inflammatory environment within the pulmonary system [14]. Furthermore, IL-4 inhibits both the trafficking of CD4+ T cells that secrete IFN-γ and the overall polarization of T cells to a Th1 or pro-inflammatory phenotype [15]. The overall anti-inflammatory environment created by IL-4 coupled with this cytokine's potential suppression of Th1 cell recruitment could dramatically alter the resident pulmonary alveolar macrophages' phenotype and enhance macrophage susceptibility to become infected by *R. equi* while decreasing macrophage functionality through dampened production of reactive oxygen intermediates.

Given that IL-4 concentrations present within foal BALF significantly decreased as the foals aged, it would not be unreasonable to presume that IL-4 has a particularly important role in tissue development during the growth of a young animal while providing the initial regulatory control system for an immature organism encountering immense amounts of new antigens and immunogens on a daily basis. Both normal growth and mild inflammation as a result of daily exposure to particulate matter or antigen load encountered during the typical life of a newborn causes the need for tissue

repair [16]. The exact reason for an increase in IL-4 concentrations within BALF collected from foals remains unclear.

Of note, in the response to instilled heat-killed *R. equi* in young foals, BALF IL-4 concentrations also increased, if higher concentrations were not already present. Initially, the BALF IL-4 concentration significantly increased in response to immunogen (w1 and w8) but this differential expression pattern is absent in foals greater than six months of age which either suggests that foal pulmonary immune responses become more adult-like at six months of age, or, rather, additional optimization of immunogen instillation is needed. The need to determine the source of IL-4, both the baseline concentration of BALF IL-4 as well as the cellular source creating the increased concentrations of IL-4 occurring in response to heat-killed *R. equi*, is important to ascertain in order to identify if baseline compared to production of IL-4 following instillation of heat-killed *R. equi* arise from the same cell type as the source of these may be different. Determining both the cell type that is responsible for production of the baseline BALF IL-4 and the concentrations of BALF IL-4 following immunogen instillation are also important to understand the immunologic mechanism behind these initial and compensatory responses as they may not be secreted by the same cellular source.

The cellular composition of BALF was studied by Hostetter *et al.* 2017 (Appendix) and percentages of macrophages were significantly higher in younger foals [9]. Furthermore, there was a significant progressive decrease in the percentage of macrophages in BALF with a simultaneous progressive increase in the percentages of lymphocytes present within the BALF (Figure 2-1). Additionally, the percentages of neutrophils decreased; however, this trend was not significant. The population of

eosinophils and mast cells, both of which secrete IL-4, were characterized and were present at a very low proportion in foals less than two months of age. Over time, a trend was present of increasing percentages of mast cells and eosinophils; however, the data were not significant. Comparison of the cellular composition in concert with the cytokine changes is interesting. The primary IL-4 producing cells, i.e., lymphocytes, eosinophils and mast cells, were all at markedly low percentages within foals less than two months of age. These cellular proportion data suggest that the IL-4 BALF concentration is not being produced by lymphocytes, eosinophils or mast cells; and the source or sources of IL-4 remains unclear. Additional studies are needed to further test this notion.

Basophils are also potent producers of IL-4 and have been detected within the peripheral blood of neonatal foals [17]; however, they were not observed within the pulmonary cellular populations [9]. In BALF collected from piglets, basophils only comprised 0-1% of the cellular population [18]. Another potential source of IL-4 is the bronchial epithelial cells [12]; however, IL-4 expression by respiratory epithelial cells has not yet been investigated in horses.

Although age-related changes in IL-4 were observed, TNF-α concentrations did not change as a result of age. TNF-α is a potent pro-inflammatory cytokine and is produced chiefly by classically activated macrophages. However, unless warranted by invading pathogens, this cytokine should not be overexpressed at the risk of producing extraneous systemic inflammatory consequences. Given that the BALF was collected from healthy foals, the consistency of the TNF-α concentrations are not surprising. However, what is interesting is the increased macrophage percentage early in life coupled with a progressive decrease in this population proportion.

A potential reason for consistent BALF TNF- α concentrations in the face of a decreasing macrophage population, could be the changing phenotype of macrophages and potential epigenetic changes of pro-inflammatory genes. Early in life, high numbers of macrophages are exposed to increased concentrations of IL-4 which likely induce a regulatory macrophage phenotype. As the macrophage proportion decreases, the concentration of BALF IL-4 also decreases while the percentage of lymphocytes increases. Therefore, it is not unreasonable to speculate that the population of macrophages from foals greater than six months old is more capable of assuming a classically activated macrophage phenotype. Without knowing how the baseline concentration of BALF IL-12 and IFN-γ and how they change over time, the exposure of macrophages to pro-inflammatory cytokines other than TNF-α in unknown. Several possibilities exist that may explain the neonates bias toward an anti-inflammatory environment. Examples include decreased expression of pro-inflammatory receptors, hypermethylation of pro-inflammatory cytokine gene regions, or other epigenetic changes [16]. Overall, the BALF TNF-α concentration, particularly in response to immunogen, should be further investigated and compared to the concentration within adults.

In conclusion, this study demonstrated decreasing concentrations of IL-4 within the BALF of aging foals from the same cohort while TNF-α concentrations remained consistent. The cause for these age-related changes remains unknown warranting further investigation. Furthermore, a complete profile of cytokines including IFN-γ, IL-10, IL-13, IL-5, IL-6, IL-12 and IL-1β would enhance the understanding of the broader scope of the cytokine milieu in the lungs of aging foals. Nonetheless, these findings demonstrate higher baseline concentrations of IL-4 contained within foal BALF when compared to a

year of life. Furthermore, in response to pulmonary immunogen instillation, a pattern of increased IL-4 concentrations was observed in foals aged one- and eight- weeks-old in comparison to foals older than six months. This response in young foals exposed to immunogen provides insight for the well documented increased susceptibility of pneumonia in foals caused by *R. equi*.

Figures

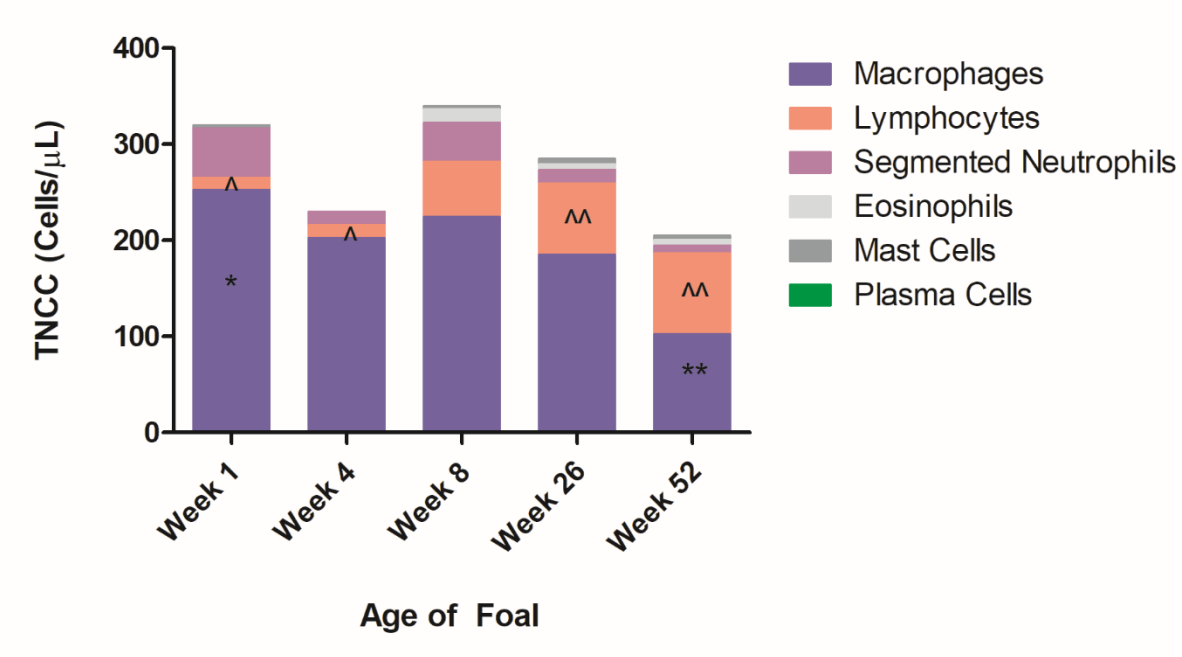

Figure 2-1. Age-related changes in the proportion of the total nucleated cell count from BALF during the first year of life in the foal. Higher TNCC counts for foals were observed at one week (median: 320/µL), four weeks (median: 230/µL), eight weeks (median: 340/µL), 26 weeks (median: 285/µL), and 52 weeks (median: 205/µL). Temporal changes in percentages of macrophages (lavender), lymphocytes (orange), neutrophils (mauve), eosinophils (light grey), and mast cells (dark grey) were observed in bronchoalveolar lavage fluid (BALF) samples taken from horses at various ages. Horses at four (*) weeks of age had a significantly higher percentage of macrophages than 52 (**) week -old horses (lavender; **$p < 0.05$). The lymphocyte percentage was lowest in horses aged one week (^) and differed significantly from 52 (^^)- and 26 (^^)-week-old horses (orange; (**$p < 0.0005$; ***$p < 0.01$, respectively). Horses aged four weeks (^) also had a significantly lower percentage of lymphocytes than horses aged 52 (^^) weeks (orange; **$p < 0.01$). Foals at one week of age had a higher median percentage of neutrophils than other age groups; however, this finding was not statistically significant (mauve). Horses at 26 weeks of age had a significantly higher percentage of eosinophils than horses aged one week and four weeks (light grey; $p < 0.01$, $p < 0.05$, respectively). Mast cells (dark grey) tended to increase over the first year of life although this trend was not significant. No plasma cells (green) were observed during the first year of life. Data are expressed as percentages of TNCC value (One-way ANOVA with Dunn's test for multiple comparisons). Figure created using modified data from Hostetter *et al.*, 2017 (Appendix) [9].

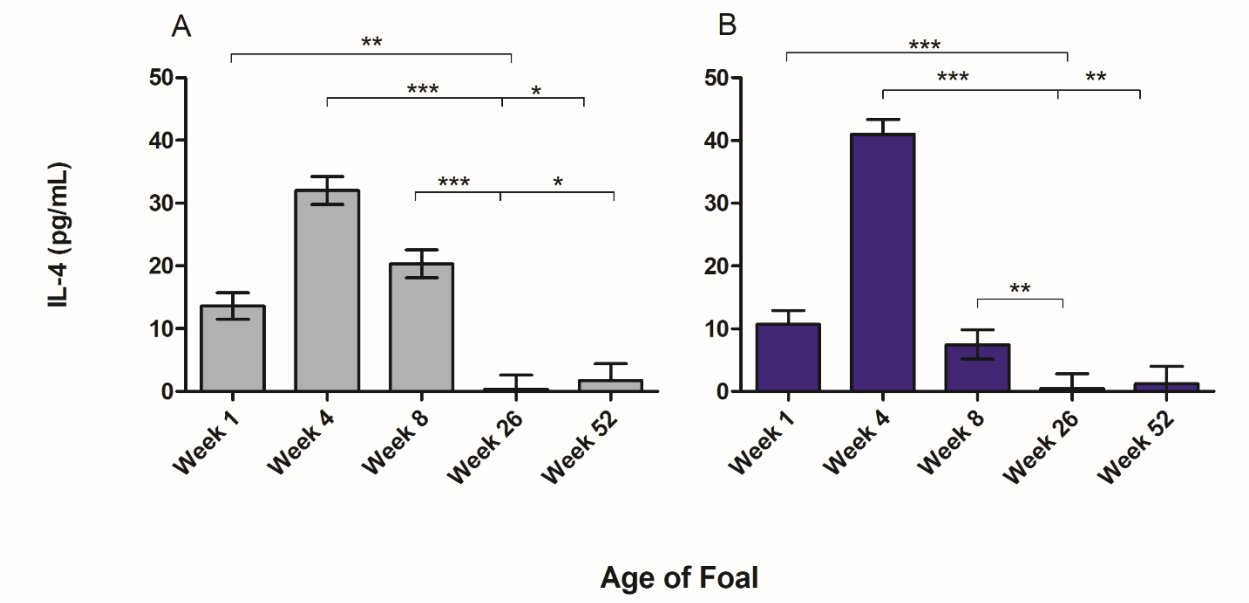

Figure 2-2. Foals have significantly greater concentrations of interleukin 4 within BALF at a young age. BALF was sampled from both the left (A) and right (B) lungs of aging foals and measured for baseline pulmonary concentrations of IL-4. IL-4 was measured by ELISA according to kit instructions (R&D Systems, Minneapolis, Mn). BALF samples were tested neat and run in triplicates; absorbance was measured at 450 nm using a fluorimeter and plotted along a 4-paramteter standard curve fit. The average concentration of IL-4 was found at each time point; the data were not normally distributed requiring a log-transformation. Data are plotted as back-transformed, normalized concentration values. Statistics were performed using the log-transformed data in an F-protected MIXED Model. Log-transformed significant differences observed within the left lung (A) are as follows: week 1 vs. week 26 ($p = 0.0012$; SEM = 0.43); week 4 vs. 26 ($p = 0.0002$, SEM = 0.44) and 52 ($p = 0.021$, SEM = 0.51); and week 8 vs. week 26 ($p=0.0007$, SEM= 0.44) and week 52 ($p=0.0467$, SEM= 0.51). Log-transformed significant differences observed within the right lung (B) are as follows: week 1 vs. week 26 ($p = 0.0008$, SEM = 0.46); week 4 vs. 26 ($p = 0.0004$, SEM = 0.47) and 52 ($p = 0.0093$, SEM = 0.54), and week 8 vs. week 26 ($p = 0.0027$, SEM = 0.47). Sample numbers are as follows: week 1, n = 7; week 4, n = 6; week 8, n = 6; week 26, n = 6; and week 52, n = 4.

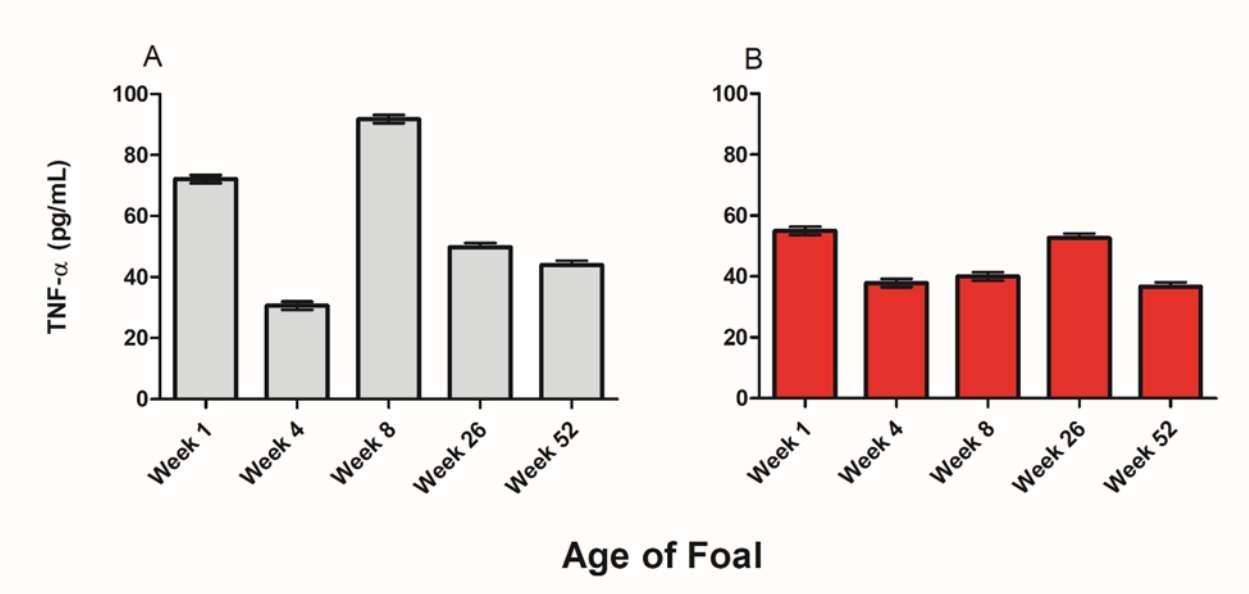

Figure 2-3. Changes of BALF TNF-α concentrations are not affected by the age of foal. BALF was sampled from both the left lungs (A) and right (B) lungs of aging foals and measured for baseline pulmonary concentrations of TNF-α. TNF-α was measured by ELISA according to kit instructions (R&D, Minneapolis, Mn). BALF samples were tested neat and run in triplicates; absorbance was measured at 450 nm using a fluorimeter and plotted along a 4-paramteter standard curve fit. Data are plotted as back-transformed, normalized concentration values. Statistics were performed using the log-transformed data in an F-protected MIXED Model. No significant differences in TNF-α levels are observed during the first year of life of the foal ($p > 0.05$ at each time point). Sample numbers are as follows: week 1, n = 7; week 4, n = 6; week 8, n = 6; week 26, n = 6; and week 52, n = 4.

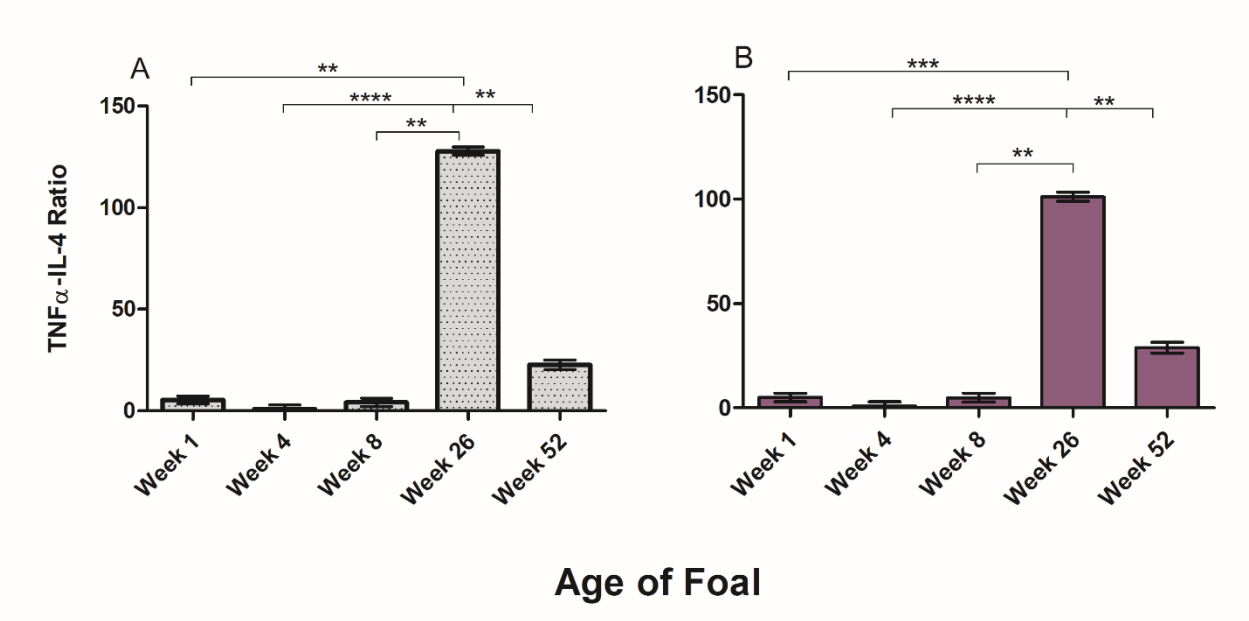

Figure 2-4. The TNF-α:IL-4 ratio significantly changed with age. Cytokines from BALF from the left lung (A) and right lung (B) were measured by ELISA according to kit instructions (R&D Systems, Minneapolis, Mn). BALF samples were tested neat and run in triplicates; absorbance was measured at 450 nm using a fluorimeter and plotted along a 4-paramteter standard curve fit. In order to normalize cytokines, a ratio was made between the pro-inflammatory cytokine, TNF-α and the regulatory/tissue repair cytokine IL-4. The change in the ratio across the first year of life is driven primarily by changes in IL-4 levels which ultimately decrease with age causing an increase in the TNF-α/IL-4 ratio at six months of age. The ratio of TNF-α:IL-4 was determined for each foal, and the ratios were then averaged, log-transformed and statistically analyzed. Data are plotted as back-transformed, normalized ratio values. Statistics were performed using the log-transformed data in an F-protected MIXED Model. Log-transformed significant differences observed within the left lung rations (A) are as follows: week 1 vs. week 26 ($p = 0.0039$; SEM = 0.44), week 4 vs. 26 ($p < 0.0001$; SEM = 0.45) and 52 ($p = 0.0098$; SEM = 0.51); and week 8 vs. week 26 ($p = 0.00287$; SEM = 0.45). Log-transformed significant differences observed within the right lung ratios (B) are as follows: week 1 vs. week 26 ($p = 0.0008$; SEM = 0.45), week 4 vs. 26 ($p < 0.0001$; SEM = 0.47) and 52 ($p = 0.006$; SEM = 0.53), and week 8 vs. week 26 ($p = 0.0084$; SEM = 0.47). Sample numbers are as follows: week 1, n = 7; week 4, n = 6; week 8, n = 6; week 26, n = 6; and week 52, n = 4.

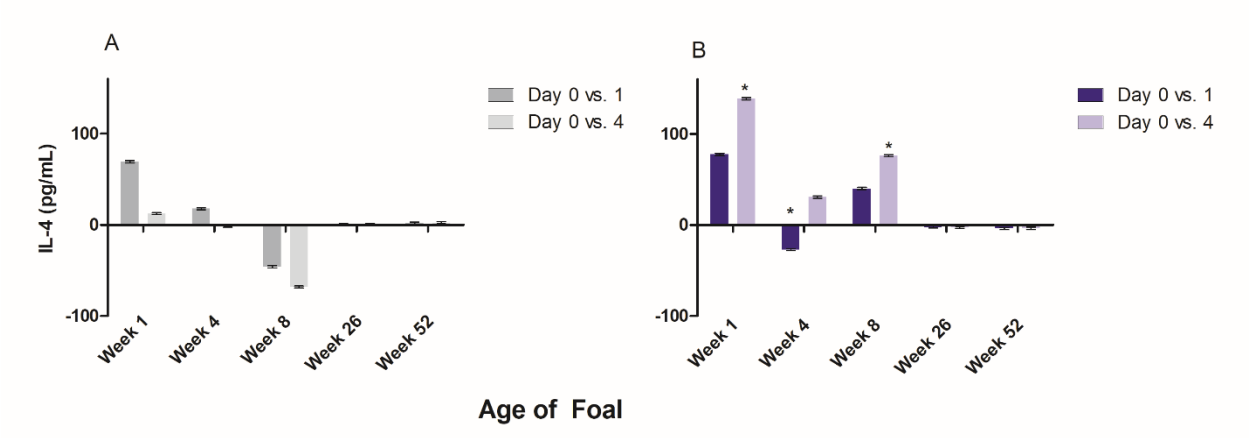

Figure 2-5. The IL-4 concentration response to heat-killed *Rhodococcus equi* vs. PBS pulmonary instillation is significantly different with age. Cytokines were measured by ELISA according to kit instructions (R&D, Minneapolis, Mn). BALF samples were tested neat and run in triplicates; absorbance was measured at 450 nm using a fluorimeter and plotted along a 4-paramteter standard curve fit. A difference between day 0 and day 1 was created by subtracting day 0 BALF IL-4 concentrations by day 1 BALF IL-4concentrations or day 4 BALF IL-4 concentrations for the left lung (A) and right lung (B). A positive bar represents an increase in the cytokine while a negative bar represents a decrease. Significant differences are present only in the right lung which received the pulmonary instilled immunogen of heat-killed *R. equi* (immunogen). During week 1, IL-4 concentrations on day 0 vs. day 4 are significantly different from each other and demonstrate a large increase in IL-4 concentrations in response to immunogen ($p = 0.0021$; SEM = 1.173). During week 4, the change in IL-4 from day 0 to day 1 demonstrates a significant decrease in IL-4 ($p = 0.048$; SEM = 1.19). During week 8, day 0 vs. day 4 demonstrates a significant increase in IL-4 in response to immunogen ($p = 0.0492$; SEM = 1.19). Sample numbers are as follows: week 1, n= 7; week 4, n=6, week 8, n=6; week 26, n=6, and week 52, n=4.

Conflict of Interest

The authors report no conflict of interest.

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CHAPTER 3. AGE-RELATED CHANGES IN MACROPHAGE MICROBICIDAL ACTIVITY IN THE FOAL

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Abstract

Pneumonia is a predominant cause of morbidity and mortality in foals less than 6 months of age; however, adult horses, unless immunocompromised, are not as susceptible to pneumonia caused by *Rhodococcus equi* . An inciting factor for the age-related susceptibility may be the extent to which microbicidal activity exists in neonatal pulmonary alveolar macrophages (PAM) and how these respective PAM are activated. A lack of effective production of nitric oxide and peroxynitrite by alveolar macrophages has been documented in severe early-onset neonatal pneumonia, indicating a defect in fundamental lung defense mechanisms during this developmental period. Given the differing pulmonary cytokine milieu between the neonate and adult horse, we speculate that macrophage phenotype is impacted by the cytokine milieu imparting varying degrees of microbicidal activity in pulmonary alveolar macrophages (PAM) and monocyte derived macrophages (MDM). To test this hypothesis, following sample collection,

peripheral blood mononuclear cells (PBMC) were cultured *in vitro* to form MDM. Then, MDM and PAM were isolated from aging foals and then stimulated and infected *in vitro* with *R. equi*. Consistent with our hypothesis, *in vitro* infected MDM and PAM derived from foals yielded a higher bacterial load than in adults. Additionally, macrophages derived from foals exhibited lower concentrations of peroxynitrite and superoxide production as compared to the adult. Cytokine profiles, including interleukin-4 (IL-4), interleukin-10 (IL-10), and interferon-gamma (IFN-γ) from stimulated PAM, were characterized by flow cytometry and yielded significant differences. The concentration of intracellular IL-4 and IFN-γ in stimulated foal PAM was greater from older foals than younger foals. However, stimulated PAM had a relative decrease in IL-10 production and a relative increase in IFN-γ and IL-4 production with age. Related to extrinsic factors directly affecting macrophage activation, CD154 (CD40L) is expressed on activated lymphocytes and functions as a co-stimulatory molecule that activates antigen presenting cells, along with cytokines and the T cell receptor interacting with the antigen being presented by the antigen presenting cell, like macrophages. CD154 was investigated and foal lymphocytes express less CD154 when compared to adult lymphocytes. Taken together, these critical differences indicate that foal macrophages are more susceptible to infection with *R. equi* and support the notion that the cytokine milieu impacts the ability of macrophages to employ a microbicidal response.

Introduction

Rhodococcus equi (*R. equi*) is a gram-positive coccobacillus bacteria that is a facultative intracellular pathogen that is phylogenetically similar to *Mycobacterium tuberculosis* causing disease in horses less than 6 months of age [1]. This bacterial

pathogen primarily causes respiratory disease in the form of chronic pyogranulomatous bronchopneumonia that is characterized by excessive abscessation; however, the disease may also occur within the enteric system where it is associated with ulcerative enterocolitis and typhlitis. Extrapulmonary disorders are common sequelae to *R. equi* infection and include other physiologic systems such as abscessation within the peritoneal cavity as well as immune-complex formation causing non-septic polysynovitis of the joints [1-3].

R. equi is ubiquitous in the farm environment, living within soil, replicating in manure, and readily being aerosolized in dust particles [4, 5]. Once the microbe gains entry into the lungs, it is readily phagocytized by pulmonary alveolar macrophages (PAM) but prevents maturation of the phagolysosome through its virulence plasmid, VapA [2, 3]. Once phagocytized, *R. equi* specifically avoids intracellular killing by the PAM through disrupting lysosome reformation; thereafter, the microbe replicates and causes massive inflammation [6]. As a result of the co-evolutionary relationship between the equine species and this bacterial species, *R. equi* is able to preferentially proliferate in foal pulmonary macrophages but not in adult equine macrophages, and this susceptibility is related to foundational immunologic differences between the foal and the adult.

A spectrum of macrophage phenotypes is recognized, and three main phenotypes are acknowledged: classically activated, wound healing, and regulatory macrophages. Interestingly, activated macrophages may be biased toward a regulatory or wound healing phenotype in neonatal foals [7, 8]. Classically activated macrophages are needed to combat *R. equi* infection due to their enhanced bactericidal functions such as the ability

to produce reactive oxygen (ROS) and nitrogen species (RONS) after engulfment of the microbe within the phagolysosome.

Professional phagocytes like macrophages produce several enzymes that, once assembled, translocate to the cellular membrane in order to catalyze highly reactive intermediates that possess antimicrobial activity [9]. Specifically, the NADPH phagocyte oxidase and nitric oxide synthase pathways are paramount for the production of superoxide and nitric oxide radicals, respectively. The reaction between superoxide and nitric oxide can form the highly reactive peroxynitrite which has broad anti-microbial activity that is crucial to microbicidal functions of classically activated macrophages [10] [11]. It has been demonstrated that cytokines and ligation of Toll-like receptors (TLR) can influence activation of macrophages and the subsequent production of reactive intermediates. In addition, ligation of TLRs can induce the expression of pro-inflammatory cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) by macrophages [2]. Therefore, our central hypothesis is that equine neonatal pulmonary alveolar macrophages produce fewer ROS and RONS due to altered macrophage activation by the pulmonary cytokine milieu of the neonatal lung.

Materials and Methods

Animals

Twelve healthy foals were sampled at one week, one month, six months, and 12 months of age for bronchoalveolar lavage fluid (BALF) and peripheral whole blood dissolved in sodium citrate. Samples were also taken from three adult geldings, greater than or equal to six years of age, or the mare of the corresponding foal.

Cell Harvest and Isolation

The bronchoalveolar lavage fluid (BALF) was collected by endoscopy using previously published techniques that were also approved by the Institutional Animal Care and Use Committee of Iowa State University [12]. A sample of the BALF was submitted to the Clinical Pathology Department at Iowa State University's College of Veterinary Medicine for assessment of cell populations. After collection, BALF was centrifuged at 500 x *g* for 30 minutes at 4° C to pellet the cellular population.

PBMC were isolated from the whole blood collected using a centrifugation technique by layering buffy coats onto a double density gradient of Histopaque-1077 and -1119 (Sigma-Aldrich, St. Louis, MO). This technique isolated the peripheral blood mononuclear cell (PBMC) population and removed granulocytes and erythrocytes from the fractions. Both BALF and PBMC cellular isolates were subsequently aliquoted into cryovials in 90% fetal bovine serum (FBS) (Atlanta Biologics, Flowery Branch, FL) and 10% DMSO (Sigma-Aldrich, St. Louis, MO) and stored in liquid nitrogen for future use. Before each experiment, aliquots from BALF and blood were thawed in 37° C water bath, suspended in 100% FBS (Atlanta Biologics, Flowery Branch, FL), centrifuged and immediately prepared for flow cytometry, or *in vitro* infection with subsequent reactive oxygen intermediate fluorimetry.

Cell Sorting

Cells from PBMC and BALF were isolated from peripheral blood and BALF, suspended in 90% FBS (Atlanta Biologics, Flowery Branch, FL) and 10% DMSO and stored in liquid nitrogen until analysis. The BALF cells were then removed from liquid nitrogen, thawed in a 37° C water bath for < 5 minutes, resuspended in Dulbecco's

Modified Eagle Medium (DMEM) (Mediatech, CellGro, Manassas, VA) with 1 g/L of glucose, L-glutamine, and sodium pyruvate without phenol red, 20% FBS (Atlanta Biologics, Flowery Branch, FL) and penicillin/streptomycin/amphotericin-B (P/S/AmpB) (Lonza, Basel Switzerland), and immediately centrifuged (500 x g for 10 minutes at 4° C). Pelleted cells were then resuspended in PBS with 0.5% bovine serum albumin and 2 mM EDTA (FACS buffer) and stained with anti-CD172a antibody (VMRD DH59B/equine CD172a, Pullman, WA) and with a secondary anti-IgG-FITC antibody (AbD Serotec, Bio-Rad Laboratories, Hercules, CA). CD172a+ live cells [13] were gated and sorted from the remaining cellular populations for infection and fluorimetry experiments and are referred to as MDM and PAM CD172a+ cells for the remainder of this chapter.

Intracellular-Bacterial Killing

PAM from either foals at week one of age (n=3) and adults (n=3) were seeded onto a 96-well clear-bottom black plate at a density of 1 x 10^5 cells/well and incubated for 24 hours at 37° C + 5% CO_2 in 100 µL of DMEM + 20% heat inactivated FBS and P/S/AmpB. Plates were then stimulated with 10 ng/mL of recombinant equine IFN-γ (reIFN-γ) (R&D Systems; Minneapolis, MN) in order to activate the macrophage enabling production of reactive intermediates for 24 hours [14]. Media was changed to DMEM +20% FBS without antibiotics and reIFN-γ. MDM and PAM were then infected with GFP and dsRed-transformed *R. equi* obtained from the laboratory of Dr. Bryan Bellaire, Iowa State University. After four hours of infection, media was changed to DMEM + 20% FBS + 50 µg/mL gentamicin sulfate (CalBioChem, San Diego, CA) to kill any extracellular bacteria that was not phagocytosed. Preliminary experiments were

carried out to ensure that *R. equi* was killed by gentamicin sulfate through plating the supernatant to confirm that no growth occurred. Infections were carried out for 24, 48 and 72 hours with media changes every 24 hours. Relative intracellular bacterial load was determined by measuring GFP (485/510) and dsRed (560/580) fluorescence using a microtiter plate reader (FLUOStar® Omega, BMG Labtech, Cary, NC). Production of superoxide and peroxynitrite was assessed by indirect fluorescent detection of oxidized dihydroethidium (DHE) or dihydrorhodamine (DHR) (Life Technologies, Carlsbad, CA), respectively, in the GFP and dsRed infected macrophages, respectively [15].

Cytokine Expression

Following thawing, PAM (n=3) were stained for CD172a, isolated via flow cytometry following live vs. dead and cell size and granularity selections. CD172a+ cells were sorted and seeded into flow tubes at 1 x 10^6 cells per tube and were subsequently stimulated with 0.5 mL of 75 µg/mL heat-killed *R. equi* for 24 hours. After stimulation, the PAM were fixed, permeabilized and stained for intracellular IL-4, IL-10, and IFN-γ (R&D Systems; Minneapolis, MN; VMRD, Pullman, WA). Flow cytometry data was analyzed by measuring the percentage of cells expressing each individual intracellular cytokine.

CD154 Expression

To investigate extrinsic macrophage mechanisms directly influencing macrophage activation, CD154 analysis of PBMC was carried out [16, 17]. PBMC from foals (n=3) at various time points (week one, month one, month six, month 12) and adult controls (n=3) were evaluated for expression of CD154. Cells were stained with a primary human

specific anti-CD154 antibody (Millipore, San Diego, CA, USA) diluted in PBS containing 20% FBS (Atlanta Biologics, Flowery Branch, FL) and washed with PBS. Then cells were stained with a rabbit F(ab')2 anti-mouse IgG:FITC secondary antibody diluted 1:100 in FACS buffer for FACS analysis. CD154+ cells were isolated via flow cytometry as described above and then seeded into flow tubes at 1 x 10^6 cells per tube and stimulated with 10 ng/ml reIFN-γ for 24 hours. After stimulation, cells were briefly washed with PBS, stained for intensity and percent population of extracellular CD154 with a human CD154-specific monoclonal mouse antibody (Millipore, San Diego, CA, USA) that demonstrated satisfactory cross-reactivity with equine CD154, per manufacturer, and a secondary rabbit anti-mouse IgG:FITC (AbD Serotec, Bio-Rad Laboratories, Hercules, CA). Then, cells were fixed with 2% paraformaldehyde for 15 minutes, washed with 4° C PBS and then resuspended in FACS buffer. Results are presented as mean fluorescence intensity (MFI).

CD154 Stimulation

Equine monocyte-derived macrophages were sorted for CD172a+ cells as described above and were seeded at 1 x 10^5 cells/well into a 96-well flat bottom plate and incubated overnight (37˚ C, 5% CO_2) in 100 µl RPMI (Mediatech, CellGro, Manassas, VA) containing 20% FBS without phenol red to avoid interference with fluorimetry readings. Plates were then co-cultured for 12 hours with 2,000 CHO cells (ATCC CCL-61TM expressing ectopic equine CD154 or without ectopic equine CD154 [18]) with or without reIFN-γ (10 ng/ml) as a positive control. DHE and DHR were used as described above. Experiments were performed in replicates of four and repeated. Data are presented as mean +/- standard deviation with significance defined as $p < 0.05$.

Statistical Analysis

Data were analyzed for normality by the Kruskal-Wallis Test. Data were then analyzed by either a one-way non-parametric (Wilcoxon Rank Test) or two-way ANOVA where applicable with Tukey's HSD post-hoc adjustments, respectively. Correlations between fluorescence of intracellular bacteria and ROS and RONS fluorescence were calculated using the Spearman correlation coefficient. Statistical analysis was performed using JMP Pro 11.0 software and SAS/STAT software (SAS Institute Inc., Cary, NC). All graphs were generated using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). For all comparisons, $p < 0.05$ was considered statistically significant. Within the figures, contrasting letters represent statistical significance.

Results

Intracellular Bacterial Killing

Pulmonary alveolar macrophages (PAM) were sorted from other cells within the BALF by their expression of CD172a (Figure 3-S1). After sorting, PAM were infected with *R. equi* transformed with either dsRed or GFP at a multiplicity of infection (MOI) of 5:1. Bacterial load was measured at 24, 48, and 72 hours post-infection (Figure 3-1A) and reactive intermediates were measured at 24 hours post-infection (Figure 3-1B1 and B2). Results demonstrated that PAM from foals infected with *R. equi* had decreased production of superoxide and peroxynitrite compared to their adult counterparts (Figure 3-1B1, $p < 0.001$ and Figure 3-1B2, $p < 0.001$). Also, foal PAM expressed higher levels of dsRed or GFP fluorescence indicating higher levels of intracellular bacteria at 48 ($p < 0.01$) and 72 hours post-infection ($p < 0.001$) (Figure 3-1A). Foal and adult PAM had a

similar infective load at 24 hours indicating the internalization of the bacteria occurred at a similar rate; however, *R. equi* was cleared from the adult PAM while it persisted within the foal PAM. An inverse correlation between the production of reactive intermediates and bacterial load was observed ($p < 0.004$) indicating a diminished respiratory burst within foal PAM, when compared to the adult, and thus, foal PAM may permit prolonged survival of intracellular *R. equi* (Figure 3-1)

Intracellular Cytokine Expression

Pulmonary alveolar macrophages from foals stimulated with heat-killed *R. equi* expressed more intracellular IL-10 and less IL-4 and IFN-γ during the first week of life ($p < 0.05$) compared to other age groups (Figure 3-2). A significant decrease in the expression of IL-10 by one month of age was observed ($p < 0.001$); however, there was still diminished expression of IL-4 and IFN-γ by PAM collected at one and six months of age compared to PAM collected at 12 months of age (IL-4, $p < 0.05$; IFN-γ, $p < 0.005$) (Figure 3-2).

CD154 Expression

Lymphocytes, isolated by size and granularity via flow cytometry (Figure 3-S1), from foals less than 12 months of age (week 1, month 1, month 2, and month 6) expressed significantly less CD154 (Figure 3-3, $p < 0.0001$). Lymphocytes recovered from foals aged 12 months had the same level of CD154 expression as their adult counterparts (Figure 3-3, $p < 0.0001$). There was no significant difference in CD154 expression on lymphocytes recovered from foals ≤ 6 months of age (week 1, month 1, and month 2).

CD154 Stimulation

In order to evaluate the ability of CD154 to stimulate macrophages, PBMC from foals within their first week of life and their dams (adult) were isolated, cultured to created monocyte-derived macrophages (MDM), and then, stimulated with a CHO cell line that had been previously transfected to express equine CD154 (Figure 3-4) [18]. Co-culture with CD154-expressing CHO cells resulted in an increase in reactive oxygen intermediates in adult MDM but did not stimulate foal MDM (Figure 3-4A and 3-4B, $p < 0.05$). Adult MDM produced significantly more superoxide (Figure 3-4A, $p < 0.0001$) when stimulated compared to foals; adults also had higher production of peroxynitrite as compared to foals (Figure 3-4B, $p < 0.0001$).

Discussion

In this study, we speculated that potential differences in the pulmonary cytokine environment within the neonatal vs. adult horse would affect the phenotype and function of the resident macrophage population. Given that foals produce more IL-4 within their pulmonary environment (Chapter 2) during their first few months of life, their macrophages are presumably biased to a regulatory or anti-inflammatory phenotype compared to an enhanced microbicidal phenotype adopted later in life. Therefore, it appears that foal macrophages evolve to a classically activated phenotype as they age and, as a result, are less likely to become infected with *Rhodococcus equi*. Furthermore, foals have a significantly greater percentage of macrophages within their BALF than adults whose BALF is predominantly composed of lymphocytes [19]. This overrepresentation of macrophages coupled with higher concentration of IL-4 exposure likely increases the probability of infection with *R. equi*. These conditions create an

excellent environment for this macrophage-tropic organism to exploit the host: increased numbers of regulatory/tissue repair macrophages that are less able to produce reactive intermediates at adequate levels to kill *R. equi*.

To test this hypothesis, foal and adult macrophages were infected with transformed *R. equi* expressing either dsRed or GFP. Bacterial load was quantified via fluorimetry. Following the foal and adult macrophage *R. equi* infection incubation period, the bacterial load in foal macrophages was significantly greater than when compared to adults (Figure 3-1). This indicates a greater susceptibility for infection within the foal macrophages compared to the adult macrophage. Furthermore, to test the hypothesis that foal macrophages produce less ROS and RONS than adults, we measured reactive intermediate production from PAM in the foal and the adult after infection with *R. equi* and following *in vitro* stimulation.

Interestingly, there was a strong inverse correlation between ROS and RONS production and bacterial load for the foal; i.e., the foal macrophages contained a higher bacterial load and lower reactive oxygen intermediates simultaneously when compared to infected PAM from adults (Figure 3-1). After 24-48 hours post-infection, the levels of superoxide and peroxynitrite were higher in the adult vs. foal macrophages (Figure 3-1B1 and 3-1B2). These data demonstrate a reduced functionality, as a result of significantly lower levels of reactive intermediates of the foal macrophage. These observations are also consistent with porcine literature. Dickie *et al.* 2009 investigated age-related changes of pulmonary alveolar macrophages in neonatal pigs and determined that ROS production from PAM significantly increased within the first week of life [20].

Given that foals contain a greater percentage of macrophages within their BALF until six months of life [20], their presumed regulatory phenotype dramatically decreases their functionality enabling *R. equi* to replicate intracellularly and cause pneumonia within foals (Chapter 2).

To investigate the inherent intracellular cytokine production, macrophages from foals and adult horses were isolated via flow cytometry (Figure 3-S1), stimulated with heat-killed *R. equi* and analyzed for presence of intracellular cytokines (Figure 3-2). Intracellular cytokine analysis revealed a higher cellular percentage of IFN-γ+ cells within the PAM population of adults and older foals, a greater percentage of IL-10+ cells within the foal PAM population compared to older foals and adult cells, and a trend of increasing IL-4+ cells in aging foals following stimulation with heat-killed *R. equi* (Figure 3-2). These data from foal PAM are consistent with previously published data on foal PBMC. Wagner *et al.* determined that foals within the first three months of life were virtually incapable of producing IL-4 following foal PBMC stimulation with PMA and ionomycin [21] while Breathnach *et al.* demonstrated that foals were IFN-γ-deficient at birth and that both IFN-γ mRNA expression as well as the secreted protein steadily increased throughout the first year of life [22].

Additionally, and consistent with our previous work demonstrating that PBMC from foals produce significantly more IL-10 mRNA than adult PBMC following stimulation with LPS or IFN-γ [23], foal PAM also produce more IL-10 than adult PAM following stimulation with heat-killed *R. equi*. These consistent data are important, especially considering the two different cell types (macrophages vs. lymphocytes) and varying methods of stimulation, and they ultimately support the notion that macrophages

from foals are biased to a regulatory phenotype and thus are less able to adopt microbicidal functionality.

Given the significant differences between the cellular population percentages within the lung of foals compared to adults as well as the inherent functional capacity difference of the macrophage, directly dependent on age, we wanted to explore whether the effects of quantitative differences in populations of cells in the lung could be further accentuated by qualitative differences in cell-cell interactions. We observed age-related differences in production of reactive intermediates by macrophages and extended our studies by focusing on the CD154/CD40 interaction between macrophages and lymphocytes. Given that lymphocytes activate macrophages during their recognition of antigen being presented by the macrophage, we investigated CD154 (CD40L) expression of lymphocytes isolated from foals. Binding of CD154, present on the surface of lymphocytes, to CD40 present on antigen presenting cells like macrophages, contributes to activation of professional phagocytes [24-26] and is an important innate mechanism of defense [18]. As previously demonstrated, without CD154, macrophage-mediated killing of both intracellular and extracellular pathogens is markedly diminished [27-29]. To characterize CD154 expression within foals and to determine the impact of this particular cell to cell interaction between the macrophage and lymphocyte, PBMC from foals and adults were isolated and analyzed for CD154 expression. Not only do foal lymphocytes express significantly less CD154 on their surface compared to adults (Figure 3-3), but foal macrophages produce significantly less superoxide and peroxynitrite as a result of CD154 stimulation when compared to the adult macrophages (Figure 3-4A, and 3-4B, respectively).

Taken together, the following results indicate that foal macrophages produce lower levels of reactive oxygen intermediates which are essential for the clearance of intracellular *R. equi*. Furthermore, and consistent with other studies, the intracellular concentration of intracellular IL-4 and IFN-γ in stimulated foal macrophages was greater from older foals than younger foals and the intracellular concentration of IL-10 was higher in younger foals [22, 23, 30]. Interestingly, stimulated PAM from older foals had a significant decrease in IL-10 production and a relative increase in IFN-γ production compared to younger foals. The influence over macrophage phenotype and functionality is multifactorial; however, patterns are present across multiple studies and support the notion that foal macrophages are biased to a regulatory phenotype and have a reduced capacity for production of reactive intermediates. Collectively, our results indicate that the pulmonary cytokine milieu may impact production of reactive intermediates in pulmonary alveolar macrophages conferring an increased susceptibility of the foal to infection by *R. equi*.

Figures

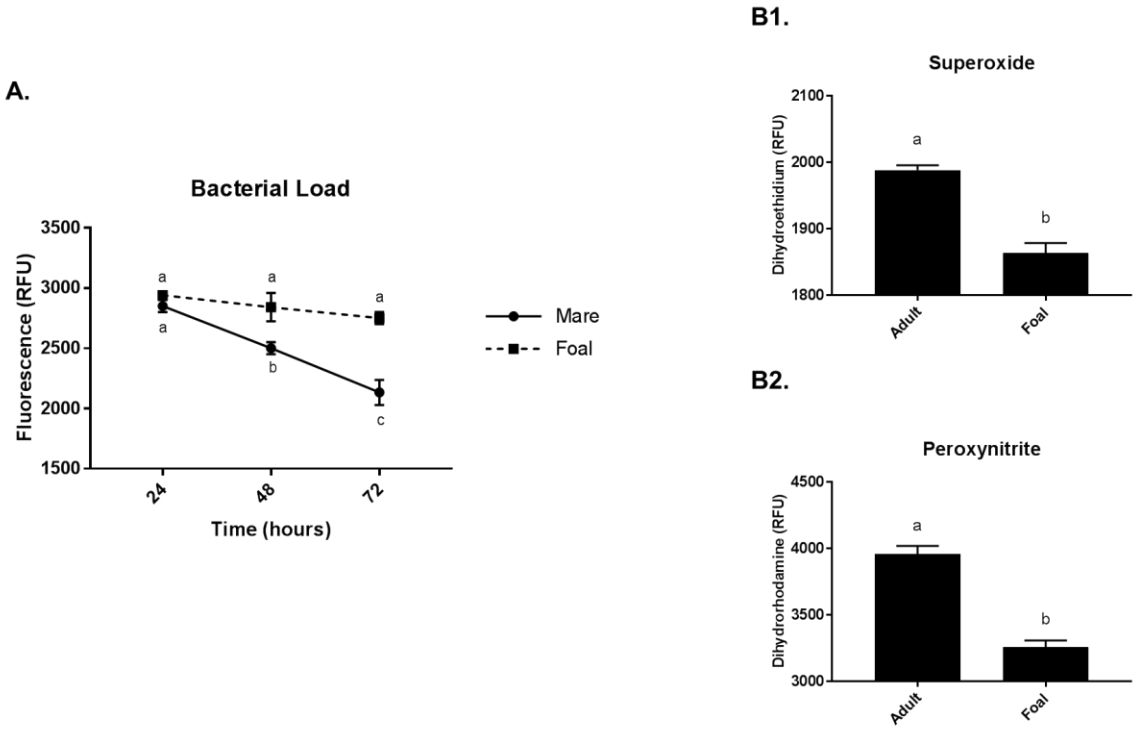


Figure 3-1. Inverse correlation of reactive intermediates and bacterial load in *R. equi* infected macrophages in foals vs. adults. In order to measure the infection rates and production of reactive intermediates, PAM were sorted based on CD172a expression and plated at 100,000 cells/well. Stimulation with and subsequent infection with GFP- or dsRed-transformed *R. equi* at an MOI of 5:1 was performed 24 hours later. One hour prior to fluorimetry readings, dihydroethidium (DHE) and dihydrorhodamine (DHR) was added to measure superoxide and peroxynitrite production, respectively. A kinetic time course (data not shown) was conducted and 30 hours +/- 6 hours was determined as the optimal level for maximum ROS and RONS production detected by both DHE and DHR. Furthermore, no difference between 0 hour and 24 RFU of the initial bacterial load was observed. Results revealed a higher production of superoxide and peroxynitrite in adult infected PAM vs. foal infected PAM (B1, $p < 0.002$; B2, $p < 0.0001$). Also, foal PAM expressed higher levels of dsRed or GFP fluorescence indicating higher levels of intracellular bacteria (A, $p < 0.002$). A Spearman correlation test was run and demonstrated a strong r-value of for DHE vs. bacterial load and a strong r-value for DHR vs. bacterial load particularly at 48- and 72-hours post-infection. Correlation coefficients and p-vales for DHE are as follows: ($p = 0.1193$; $r = 0.493$); 48 hours post-infection ($p = 0.0257$; $r = 0.752$); and 72 hours post-infection ($p = 0.0038$; $r = .0902$). Correlation coefficients and p-values for DHR are as follows: x 24 hours post-infection ($p = 0.0616$; $r = 0.624$) ($p = 0.0079$; $r = 0.858$); and 72 hours post-infection ($p < 0.0001$; $r = 0.991$).

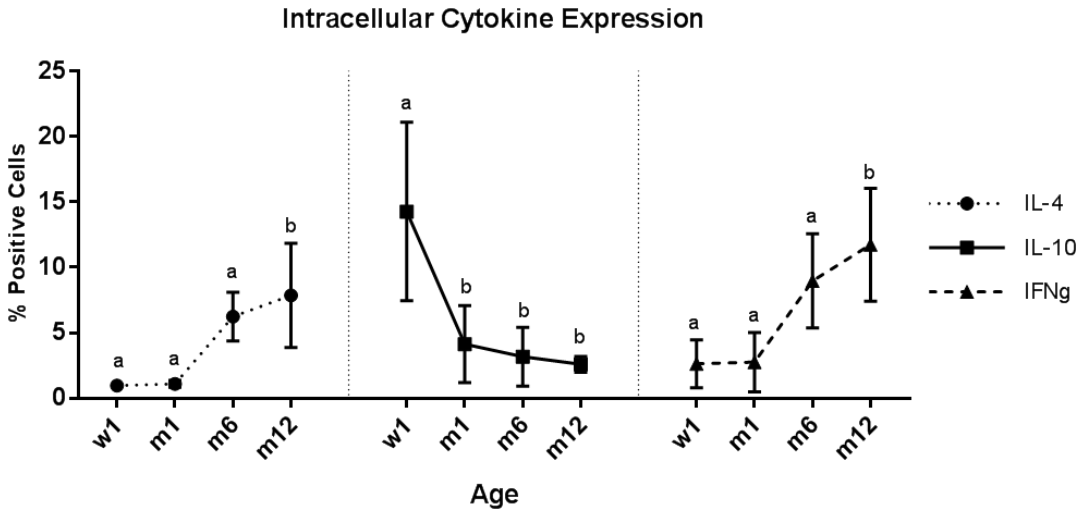


Figure 3-2. Macrophages from younger foals express elevated levels of IL-10 and decreased levels of IFN-γ and IL-4. Pulmonary alveolar macrophages sorted by CD172a selection were seeded into FACS tubes at 1 x 10^6 cells/tube and stimulated with 0.5 mL of 75 µg/mL of heat-killed *R. equi* antigen. After 24 hours cells were fixed, permeabilized and stained for intracellular IFN-γ, IL-4 and IL-10. Results are expressed as the percentage of cells positive for each cytokine. PAM express more IL-10 and less IL-4 and IFN-γ during the first week of life ($p < 0.01$). There is a decrease in the expression of IL-10 by month 1; however, there is still diminished expression of IL-4 and IFN-γ in foal PAM at month 1 of age compared to month 6 and month 12 ($p < 0.05$).

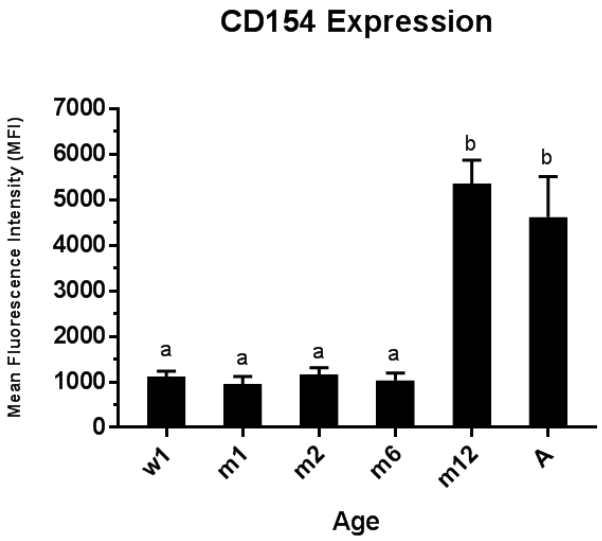


Figure 3-3. Lymphocytes from foals less than twelve months of age express significantly less CD154 compared to adult lymphocytes. PBMC from foals from various time points and three adult controls were seeded into FACS tubes at 1 x 10^6 cells/tube and stimulated with equine reIFN-γ overnight. Cells were stained with primary mouse anti-human-CD154 antibodies and a secondary rabbit anti-mouse FITC antibody and then fixed with 2% paraformaldehyde. Lymphocytes were gated based on size according to Figure 3-S1. The graph above shows the mean fluorescence intensity of the lymphocyte gated cells representing the CD154 positive lymphocytes. There is a significant increase in the CD154 expression at month 12 of age ($p < 0.0001$) which is comparable to adult CD154 expression levels.

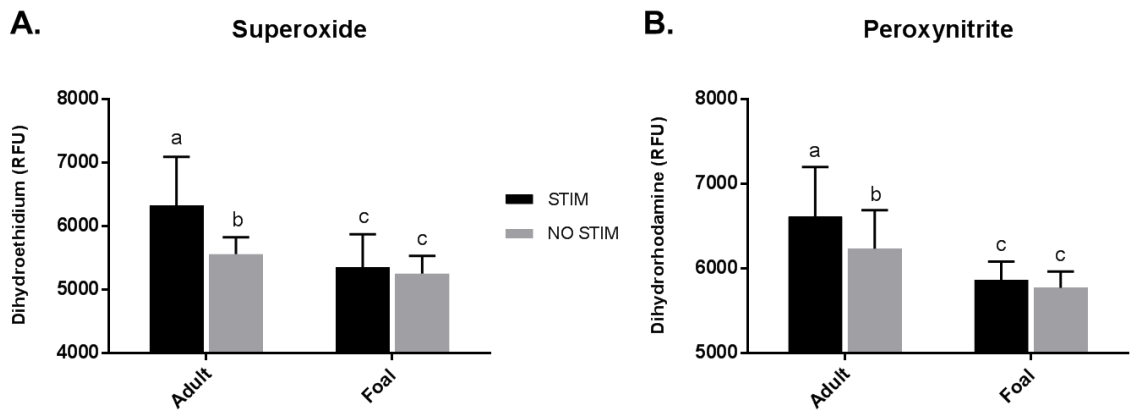
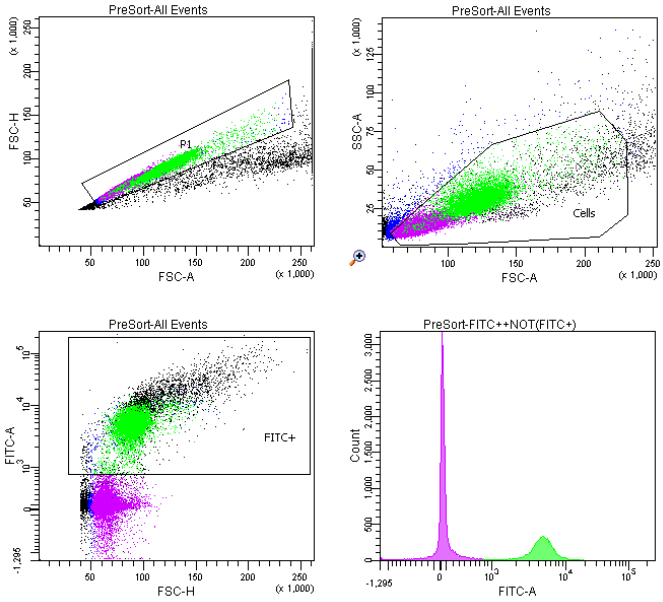


Figure 3-4. Macrophages from foals stimulated with CD154 expressing cells display diminished production of reactive intermediates in comparison with their adult counterparts. Monocyte-derived macrophages sorted by CD172a+ were selected from foals less than 1 week of age and adults (the dams of each foal) were cocultured with CHO cells that were either expressing the ectopic equine CD154 (stim) or were not expressing the ectopic equine CD154 (no stim). Cells were considered stimulated cells if were from co-cultured with equine ectopic CD154 expressing CHO cells, and non-stimulated cells were those co-cultured with CHO cells that did not express CD154. Dihydroethidium (detecting superoxide) and dihydrorhodamine (detecting peroxynitrite) were used to measure reactive intermediate response. Foals displayed lowered production of reactive intermediate with or without stimulation with CD154 expressing CHO cells compared to their adult counterparts ($p < 0.0001$).



Supplemental Figure 3-S1. Efficient purification of macrophages from PBMC and BALF cellular populations using a CD172a sorting protocol via flow cytometry. PBMC and BALF-derived PAM were removed from liquid nitrogen and immediately thawed. PBMC were cultured overnight to form MDM. Then MDM and PAM cells were then stained with anti-CD172a antibody and with a secondary rabbit anti-mouse IgG-FITC antibody. Single cells were selected based on the P1 gate (top left). Dead cells and debris were removed (top right) from the population and the remaining live cells were gated based on size and granularity (bottom left) similar to cells of monocyte lineage (green population). Finally, cells were gated based on their expression of CD172a+ (green population in bottom left). Cells that met all three gating parameters were sorted and kept for testing (bottom right). The same gating strategy was used for macrophages within the PBMC and BALF cellular populations. Approximately 20% of the cells in the original population were CD172a+ for MDM and 80% for PAM, respectively. Lymphocytes (pink population) were negatively selected using the same gating strategy but were CD172-.

Conflict of Interest

The authors report no conflict of interest.

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CHAPTER 4. AGE-RELATED CHANGES IN THE CONCENRATION OF CIRCULATING SOLUBLE IMMUNE COMPLEXES AND THEIR INFLUENCE ON MACROPHAGE FUNCTION IN THE HORSE

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Abstract

While antimicrobial drugs remain the most common approach for treatment of intracellular infections, the increasing emergence of multidrug-resistant-organisms requires the need for alternative methods of antimicrobial activity. One spontaneous, naturally occurring bacterial disease model is pneumonia caused by *Rhodococcus equi* which is common in foals less than six months of age and immunocompromised humans. *R. equi* is a common and devastating pathogen frequently causing high morbidity and mortality from severe bronchopneumonia in young foals; however, immunocompetent adult horses are not susceptible to this intracellular pathogen. Given the age-related susceptibility to disease in neonates, the need for alternative antimicrobial therapy is imperative. Evidence demonstrates that plasma products reduce the incidence and severity of pneumonia in foals but the mechanism largely remains unknown. We speculate that soluble immune complexes (sIC), present in plasma products, interact with

surface Fc-gamma receptors (FcɣR) on macrophages and may contribute to the success of equine plasma products in the prevention and decreased severity of clinical *R. equi* pneumonia. Our study suggests that non-specific activation of macrophage FcɣR by sIC results in increased RONS. Furthermore, the circulating sIC concentration within neonates is significantly lower than adults supporting speculation of the potential mechanism behind prophylactic treatment of hyperimmune plasma. Importantly, a previous study demonstrated a reduction in the *in vitro* load of another macrophage-tropic intracellular pathogen, *Leishmania amazonensis,* through sIC stimulation of infected macrophages. These results support our hypothesis that soluble immune complexes activate macrophages via the FcɣR resulting in intracellular killing of pathogens. It is plausible that these non-specific mechanisms apply to other macrophage-tropic intracellular pathogens.

Introduction

Rhodococcus equi is a gram-positive coccobacillus bacterium that causes pneumonia in foals less than 6 months of age. There are multiple mechanisms imparted by the virulence plasmid to enhance virulence including those that evade the phagolysosome. Neonatal pulmonary alveolar macrophages (PAM) have been identified as deficient in their production of superoxide and peroxynitrite contributing to fulminant early-onset pneumonia[1]. This indicates a defect in fundamental lung defense mechanisms during this developmental period. A potential mechanism that can be used to enhance the host immune response to intracellular bacterial infections involves modulating the interaction between Fc gamma receptors (FcɣR) on the surface of macrophages, including PAM, with naturally circulating soluble immune complexes

(sIC). Expressed on the surface of macrophages, ligation of FcγR aid in phagocytosis and superoxide production [2]; both of these macrophage functions are required for killing of intracellular pathogens like *R. equi* [3]. Previous studies have demonstrated that the interaction of immune complexes (IC) with FcγR expressed on the surface of macrophages results in antibody-dependent cellular cytotoxicity (ADCC), phagocytosis and superoxide generation [2]. Furthermore, clinical research has demonstrated that plasma products, not only limited to *R. equi* specific hyperimmune plasma, reduce the incidence and severity of pneumonia caused by *R. equi* in foals [4-10] but the precise mechanism remains unknown. We speculate that foals have less circulating sIC than adult horses and that sIC, present within plasma products, provide protection to foals by interacting with surface FcγR and contribute to the protective effect of equine plasma products.

In this study, sIC were isolated from the serum of neonatal and adult horses and their concentrations were compared using a commercial kit turbidometric assay. The sIC were also measured for protein content and tested for specificity with an anti-equine IgG antibody through western blot analysis. The *in vitro* biologic role of macrophage activation through stimulation with sIC was investigated through ROS fluorometric assays. Building upon a previously identified mechanism using sIC to enhance intracellular killing of pathogens like *R. equi* through the upregulation of reactive intermediates, intracellular killing of *R. equi* was quantified. Through the use of our well-established *in vitro* protocol utilizing PAM, we have demonstrated the ability to enhance superoxide production through the *in vitro* stimulation of macrophages with naturally occurring circulating soluble immune complexes. We speculate that sIC, which

are comprised of circulating immunoglobulin, mainly IgG and antigen, interact with surface FcγR on macrophages. Ligation of and signal transduction by FcγR expressed on the surface of macrophages initiate phagocytosis and superoxide production, a common mechanism for killing of intracellular pathogens [2]. Our preliminary data suggest that non-specific activation of macrophage FcγR by sIC result in increased ROS production. Our goals are to characterize the concentration of circulating sIC within neonates compared to adults and to investigate sIC ability to upregulate ROS. The results of this study are expected to provide insight into alternative methods of bacterial control for diseases affecting both animals and humans.

Materials and Methods

Sample Collection

Serum samples were collected via venipuncture in red top tubes (RTT) from foals and adult horses upon admission to the Iowa State University Lloyd Veterinary Medical Center after receiving the Iowa State University Institutional Animal Care and Use Committee (IACUC) approval as well as the owner's informed consent. After proper clot formation, the sera samples were centrifuged for 10 minutes and the serum was aspirated and stored at -20° C for future analysis. Bronchoalveolar lavages (BAL) were performed on adult horses as previously described [11]. Adult horses were sedated with intravenous detomidine (0.01 mg/kg) and butorphanol (0.01 mg/kg) prior to the procedure. A sterilized Olympus VQ-8303A (9 mm OD) video-endoscope was used to identify the airway of interest. Mepivicaine (2%) was instilled during advancement of the endoscope to limit coughing and discomfort. Lavages were performed using 480 mL of sterile saline

in 60 mL aliquots in the adult. Similarly, a sterilized Olympus GIF-XP160 280 (5.9 mm OD) pediatric videoendoscope was used with 280 mL of sterile saline in 35 mL aliquots for obtaining bronchoalveolar lavages from heavily sedated foals (0.8 mg/kg xylazine IV; 5.0 mg butorphanol IV). After insertion of the endoscope into the elected site, cells were harvested in syringes by aspiration of the instilled saline.

Remaining fluid was placed into 50 mL conical tubes and centrifuged at 500 x *g* for 10 minutes at 4° C. Pellets were washed using PBS (Mediatech, CellGro, Inc., Manassas, VA) containing decreasing amounts of antibiotic/antimycotic (Lonza, Morristown, NJ) (wash 1: 2.5 µg amphotericin-b/mL; wash 2: 0.1 µg amphotericin-b/mL, 2 U of penicillin/mL, and 2 U streptomycin/mL). Cells were pelleted after wash 2 and resuspended in freezing medium containing 90% fetal bovine serum (FBS) (Atlanta Biologicals Inc., Flowery Branch, GA) and 10% DMSO (Sigma-Aldrich, St. Louis, MO). Cells were placed in an isopropanol Mr. Frosty® freezing container (Nalgene, Penfield, NY) at -80° C for 24-72 hours prior to transfer to liquid nitrogen.

Soluble Immune Complex (sIC) Isolation via PEG6000 Precipitation

Serum samples were thawed and immediately mixed with an equal volume of 5% PEG 6000 with 0.1 M HEPES (Hampton Research Corp., Aliso Viejo, CA) and left to stand at 4° C overnight before the precipitates were purified and washed using a modified protocol from a previously described method [12, 13]. Briefly, after overnight incubation, the sera:PEG sample was centrifuged at 2,100 x *g* at 4° C for 20 minutes to pellet the sIC and the remaining serum was either discarded or saved for further analysis. The pelleted precipitates were reconstituted with ice-cold sterile PBS (Mediatech

CellGro, Inc., Manassas, VA) to the original volume of serum. The PEG precipitates were stored at 4° C until used in cell culture experiments.

Immunoglobulin, sIC and Protein Concentration Analysis

The protein concentrations from the sIC sample and the sera that it was derived from were analyzed using the BCA assay (Pierce Technologies, Waltham, MA) as well as the Nanodrop1000 (Thermo Scientific, Waltham, MA). The immunoglobulin concentration of the serum and respective sIC were tested using a turbidimetric immunoassay read at 530 nm (DVM Rapid Test II®, Value Diagnostics, Sarasota, FL). The test result is reported as mg/dL.

Western Blot Analysis

Samples containing sIC isolated from serum were quantified using a NanoDrop, and 10 ng of total protein from each sample were added to appropriate wells of a NuPage 4-12% Bis-Tris Gel (ThermoScientific, Waltham, MA). The gel was run according to standard denaturing western blot procedures [14]. Briefly, the gel was run using a Bis-Tris buffer with an SDS denaturing incubation step prior to loading the samples onto the gel. The resulting gel was then transferred to a nitrocellulose membrane, blocked, and incubated with a goat-anti-horse IgG antibody conjugated with an HRP tag (KPL, SeraCare, Milford, MA) used for visualization.

Bacterial Growth and Concentration Determination

Rhodococcus equi (ATCC 33701) was streaked from glycerol stocks onto a TSA-II plate (BD, Franklin Lakes, NJ) and incubated at 37° C, 5% CO_2 for 48 hours. A single

colony was then streaked for isolation and incubated at 37° C, 5% CO_2 for 36 hours on TSAII plate (BD, Franklin Lakes, NJ). All growth was removed from the 36-hour growth plate using a sterile loop and the colonies were placed into 10 mL of sterile BHI broth (BD, Franklin Lakes, NJ) and shaken at 37° C, 5% CO_2 for 15 minutes in order to homogenize the broth. A dilution yielding an OD_{600} = 0.300 was recovered, which corresponded to a bacterial concentration of approximately 5.5 x 10^8 CFU/mL, for infection of macrophages.

R. equi Macrophage Infection and Lyse and Plate Protocol

Murine macrophages (RAW264.7) were plated at a density of 100,000/well in a 96 well plate and incubated at 37° C, 5% CO_2 overnight and subsequently infected with *R. equi* (ATCC 33701; and Strain T194, ISU Clinical Isolate) with an MOI of 200:1 for 3 hours at 37° C, 5% CO_2. The cells were then washed with 100 µL of warm PBS and media with 50 µg/mL of geneticin (CalBioChem, San Diego, CA) was applied to the cells in order to kill extracellular bacteria. The cells were then incubated for 24 hours at 37° C, 5% CO_2. The next day, the supernatant was removed and a 0.05% Triton X-100 (ThermoScientific, Waltham, MA) solution was applied to the cells for 5 minutes. Ten-fold serial dilutions were performed and plated for visual quantification following a 24-72-hour incubation at 37° C, 5% CO_2.

Statistical Analysis

Sample means were found for the sIC, IgG, protein and ROS values using the statistical software package, GraphPad Prism 5 (La Jolla, CA). For respective populations, a T test or paired T test, where appropriate, was conducted and graphs were

generated using GraphPadPrism 5 software (La Jolla, CA). Statistical significance was accepted at a p-value < 0.05.

Results

Foals Have Significantly Less Circulating sIC than Adult Horses

Soluble immune complexes were isolated from the blood of neonatal and adult horses using a PEG precipitation method as described above. The immunoglobulin and sIC concentrations were measured using a turbidometric method according the manufacturer's instructions (DVM Rapid Test II®, Value Diagnostics, Sarasota, FL). Neonatal foals do not have any circulating sIC prior to colostrum ingestion (Figure 4-1). Following colostrum ingestion, neonatal horses have significantly less circulating sIC ($p < 0.0001$) when compared to adult horses (Figure 4-2).

The Ratios of Immunoglobulin and Protein within sIC are Significantly Different Between the Foal and Adult

Soluble immune complexes are composed of immunoglobulin and their cognate antigen or protein. Concerning the composition of the sIC from foals, foals have both less protein content and immunoglobulin content within their sIC concentrations. When comparing IgG content within serum to IgG content within the sIC that was isolated from the serum, the foals have higher ratios because their IgG and protein concentrations are significantly lower within their sIC than in their sera (Figure 4-3A; 4-3B). Given that following colostrum intake, the serum concentration of IgG does not significantly differ between foals and adults (data not shown, $p > 0.1$), the difference is in the number of sIC

within their circulation as well as the protein, likely antigen, content within those sIC (Figure 4-3, $p < 0.01$). However, foals have a lower range of normal serum protein concentrations when compared to adult horses so their higher protein ratio is not unexpected [15, 16].

Soluble Immune Complexes are Specific for Equine Immunoglobulin

Immunoglobulin molecules are approximately 150 kDa in size while sIC are larger and measure approximately >150 kDa[17] . Preliminary western blot analysis demonstrates the presence of equine IgG within the isolated sIC (Figure 4-4). Furthermore, the western blot image shows the IgG heavy chain and light chain at 75 kDa and 28 kDa, respectively (Figure 4-4). The precipitation of sIC via the PEG precipitation method is based on aggregation of sIC on a charge and size basis and is relatively selective for immune complexes; however, this method may also precipitate other high molecular weight proteins as previously mentioned [18, 19]. In order to confirm specificity of the anti-equine IgG antibody, ovalbumin and murine IgG were loaded as a negative control and did not display any banding pattern (data not shown).

Macrophage Stimulation with sIC Results in an Increase in ROS Production

The biological activity of the isolated equine sIC was investigated through *in vitro* ROS studies. Two macrophage sources were used to investigate production of ROS as a result of sIC macrophage stimulation. RAW264.7 were plated at a density of 100,000 cells/well in a 96-well black plate. They were rested overnight, stimulated with 50-100 µg/mL of equine sIC, isolated from three different adult horses, and simultaneously

stimulated with 10 ng/mL of recombinant IFN-γ (R&D Systems, Minneapolis, MN) for 72 hours. Following 72 hours of stimulation, DHE (25 μM) (Life Technologies, CA) was incubated and a fluorimetry reading at 544/620 nm excitation/emission spectrum, respectively, was obtained following removal of the supernatant. Figure 4-5A demonstrates that following stimulation with IFN-γ and sIC, macrophages significantly increase their production of reactive oxygen species. The sIC from these three adults were each tested in two separate experiments. For the second macrophage source, equine PAM were isolated using culture adherence techniques following a BAL, from adult horses and were plated at a density of 50,000 cells/well and allowed to rest overnight at 37° C, 5% CO_2. The following day, the PAM were stimulated with 10 ng/mL of equine IFN-γ and 50-100 μg/mL of equine sIC isolated from donor horse serum (DHS) (Mediatech, CellGro, Manassas, VA). Following 72 hours of stimulation, DHE (25 μM) was incubated and a fluorimetry reading at 544/620 nm excitation/emission spectrum, respectively, was obtained following removal of the supernatant. Compared to the unstimulated PAM, the PAM that were stimulated with the DHS sIC showed approximately 2.5 times more ROS production (Figure 4-5B). A positive control of synthetically made sIC (equine IgG and ovalbumin) resulted in a two-fold increase in ROS production compared to the unstimulated cells (Figure 4-5B). Although these results were not significant, a trend consistent with the previous data can be observed. Due to limited quantities of primary equine pulmonary alveolar macrophages, this experiment was not repeated.

Discussion

Soluble immune complexes and their function within the body have previously been studied in the context of autoimmune diseases such as in patients with rheumatoid arthritis and systemic lupus. In these diseases, the mechanism of action of both soluble and insoluble immune complexes exacerbates inflammation due to production of reactive oxygen species and/or release of peroxidase enzymes making these immune complexes undesirable in these patients. Conversely, though undesirable in autoimmune disease, their presence may play a role providing protection against infections; however, this has been largely overlooked during physiologic states of health and the literature exploring this concept is lacking. In this study, a potential mechanism of action regarding the prophylactic effect of hyperimmune serum has been proposed. A decrease in the incidence and severity of pneumonia in foals has been clinically demonstrated through the prophylactic use of plasma products [4-10] but results are controversial and the mechanism largely remains unknown other than success has been observed independent of the source of plasma, i.e., *Clostridial spp*. *Streptococcus spp*. vs. *Rhodococcus equi* specific hyperimmune plasma. We speculate that sIC present within hyperimmune plasma products interact with surface Fc gamma receptors (FcγR) on macrophages and may contribute to the success of these equine plasma products by aiding in ADCC, phagocytosis and superoxide production [2]. In the context of intracellular killing of pathogens, these mechanisms are not only favored but required for killing of intracellular pathogens like *R. equi* [3].

For this study, soluble immune complexes were isolated from foals and adults using a previously published method [13]. Circulating soluble immune complexes were found at significantly lower concentrations than sIC within the adult circulation. Foals

rely mainly on maternal antibody acquired by passive transfer through ingestion of colostrum. Although limited in isotypes, an array of endogenous antibody production including IgG1, IgG3, IgG5 and IgA within the foal matures by three months of age but it is not until one year of age that all immunoglobulin-G antibody isotypes including IgG4, IgG7 as well as IgE can be isolated [20]. Thus, the lack of environmental exposure of antigen coupled with a limited quantity of circulating antibody would be predicted to result in lower systemic concentrations of sIC (Figure 4-1, $p < 0.05$). Furthermore, due to a lack of environmental exposure, it is possible that the circulating immunoglobulins received from passive immunity remain uncomplexed. In other words, the immunoglobulins present in neonates are free antibodies and are not complexed with their cognate antigen rendering them unable to cross-link FcγR on the surface of macrophages and signal through the immunoreceptor tyrosine-based activation motif (ITAM). It is also plausible that what sIC are present post-ingestion of colostrum originate from the dam.

Here we investigated the concentration of sIC within neonates and adults as well as their ability to activate macrophages through ROS quantification. It should be stated that great variability was observed when testing sIC from various adults specifically in the *in vitro* macrophage stimulation ROS assays. A potential reason for this variability may include the various immunoglobulin isotypes present within the sIC and their varied interactions with FcγR, as not all isotypes signal through ITAM activation, as well as the amount and type of antigen or protein present. For future directions, complete characterization of the sIC is needed in order to determine the isotype of immunoglobulin present within those isolated sIC, as well as the amount of antigen or protein present and

if there are other serum proteins that aggregated during the PEG incubation as well as the associated variation between individuals. Concerning the formation and composition of IC *in vivo,* individual variation is vast making repeated isolations from different equine patients and subsequent use during *in vitro* assays challenging. For instance, Fossati *et al.* states that when making synthetic soluble immune complexes in the laboratory, 120 µg/ml of antigen in concert with 80 µg of antibody is needed; if not enough antigen is present then insoluble complexes are formed when antigen concentrations reach only 20 µg/ml [21]. The variability due to the antibody isotype and zone of equivalence (ratio of antigen to antibody) can greatly impact the functionality of the IC stimulation [21]. For this reason, a comprehensive characterization is needed following isolation of sIC from subjects. Subsequent to a thorough and repeatable sIC characterization, sIC can be used in order to study their intracellular killing abilities after incubation with macrophages infected with *R. equi*. This observed variability could also be the reason behind the controversial results observed with equine hyperimmune plasma [22]. Concerning VapA specific IgG isotypes, Sanz *et al.* observed coefficients of variance between 17 and 123 per cent between various lots and manufacturers of hyperimmune plasma product [22].

In order to better assess sIC as an alternative antimicrobial therapy, the macrophage (RAW264.7) infection and stimulation with IFN-γ and sIC protocol needs to be optimized as these experiments were repeated several times and yielded equivocal results. Prior to forming firm conclusions regarding sIC upregulation of intracellular bacterial killing, the bacterial infection protocol needs optimization as well as the lyse and plate method for growth of *R. equi* and subsequent macrophage infection protocols.

Collectively, these results support the notion that soluble immune complexes activate macrophages via FcγR resulting in an increase in ROS and presumably antibody dependent killing of intracellular bacteria as previously demonstrated using *Leishmania amazonensis* [23]. The central hypothesis of the proposed experiments is that neonates have lower concentrations of sIC than adults and that these sIC upregulate bactericidal activity of pulmonary alveolar macrophages. The implications of these non-specific alternative antimicrobial therapies are broad and are expected to apply to other pathogens such as *Salmonella spp*., *Mycobacterium spp*., and *Leishmania spp*. etc. The results of this study are anticipated to provide insight into innovative strategies for novel mechanisms of bacterial control of intracellular pathogens that threaten horses or other mammalian species. Ultimately, the outcomes of future studies are expected to enable the development of alternative antimicrobial therapies, decreasing the dependency on antibiotics.

Figures

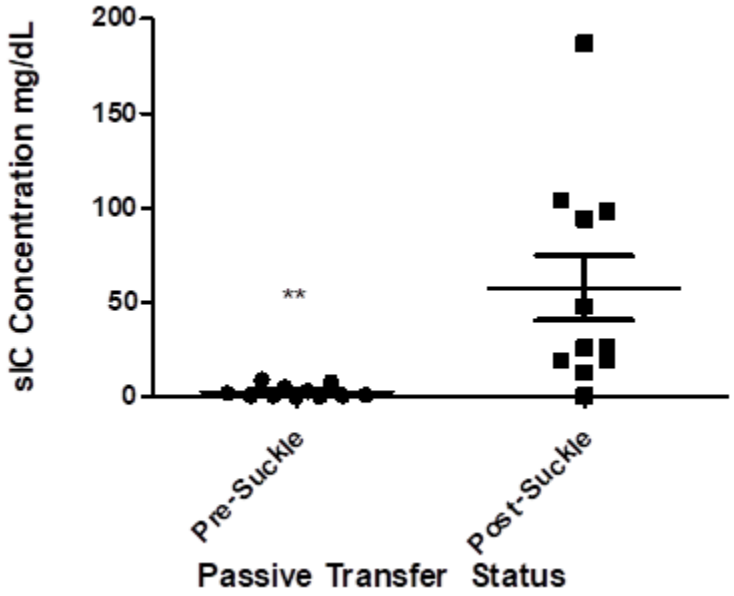

Figure 4-1. Soluble immune complex concentrations in foals before and after ingestion of colostrum. sIC were isolated using a PEG protocol and were measured using a turbidimetric immunoassay which was read at 530nm (DVM Rapid Test II, Value Diagnostics). These results demonstrate the proof of concept that sIC are not present prior to external provision of colostrum or intravenous plasma and provide an assay control for the PEG isolation of soluble immune complexes. Prior to colostrum ingestion, foals do not have any circulating immunoglobulin and therefore, no soluble immune complexes are present. Graphs and statistics were generated using GraphPad Prism 5 (GraphPad software, La Jolla, CA) using a paired T test analysis. n=12 neonatal horses. **$p < 0.001$

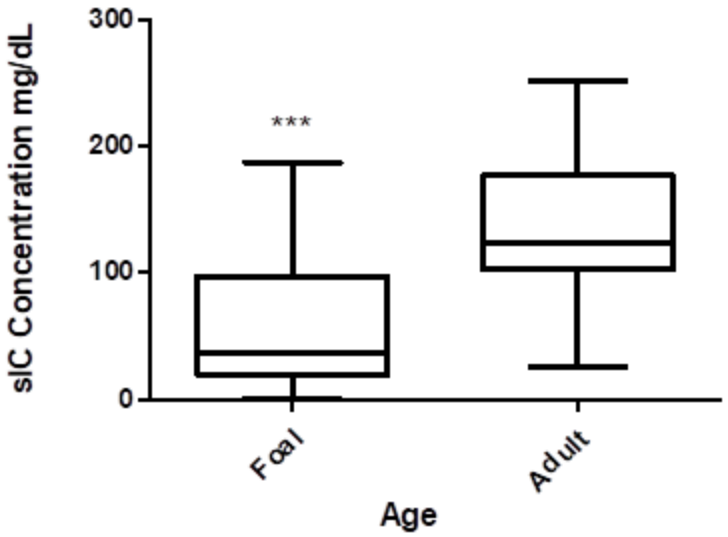

Figure 4-2. Age-related differences in soluble immune complex concentration of equine sera. sIC were isolated as described above. Results demonstrate the significantly lower concentration of sIC within neonatal sera compared to adult. n=39 horses (12 neonates, 28 adults). Data is displayed as a box and whisker plot and statistics were generated using GraphPad Prism 5 (La Jolla, CA) through a Student's T-test analysis. ***$p < 0.0001$

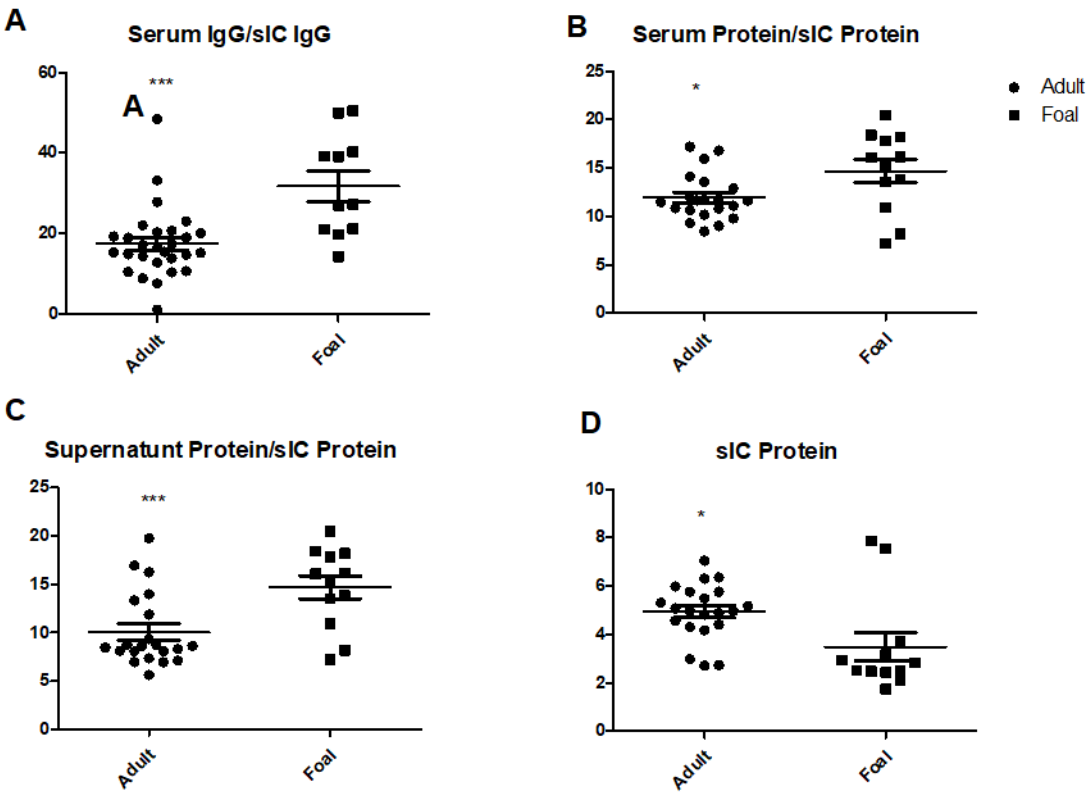

Figure 4-3. Ratios of immunoglobulin/protein concentration in serum respective to sIC concentration. sIC were isolated as described above. A ratio was made using serum IgG concentration to sIC IgG concentration. There is a higher IgG ratio in foals due to a lower sIC concentration of immunoglobulins when compared to adult ratios $p < 0.0001$(A). A similar pattern is present when comparing sera protein concentrations to protein concentrations within sIC , although given that healthy foals naturally have less serum protein than adults this is not unexpected $p < 0.01$ (B) as well as the remaining protein content within the supernatant from the PEG isolation $p < 0.0001$(C). Adults also have significantly more protein within their sIC than foals $p < 0.01$(D).

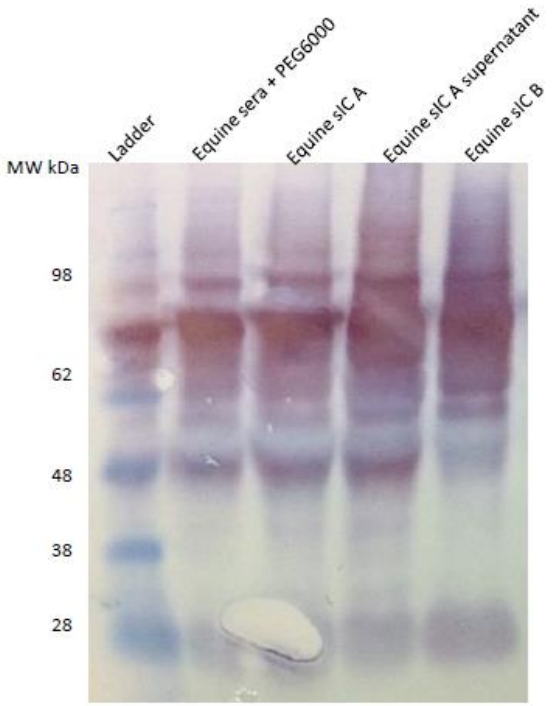

Figure 4-4. Western blot analysis of equine sIC samples. sIC were isolated from serum samples as described above. The supernatant from one of the sIC samples was run for comparison of IgG content isolated in the sIC sample. Murine sIC samples and ovalbumin were used as negative controls to confirm equine antibody specificity (data not shown). Samples of equal protein (10 ng) content were loaded into each well of a NuPage 4-12% Bis-Tris Gel (ThermoScientific, Waltham, MA), following a denaturing SDS incubation step, ran and then transferred to a nitrocellulose membrane. An equine anti-IgG-HRP antibody (KPL, SeraCare, Milford, MA) was then incubated and visualized. n= 2 adults.

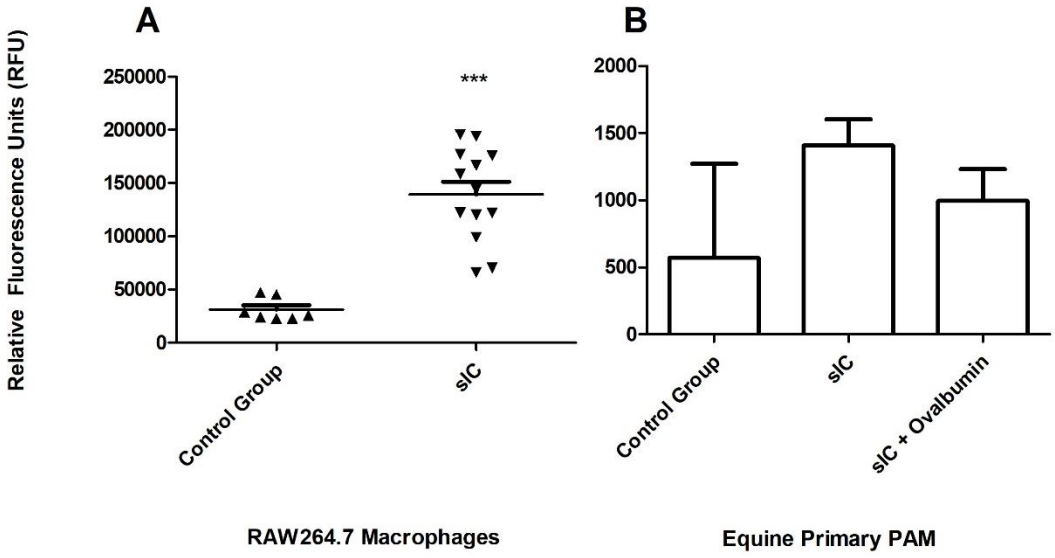

Figure 4-5. Macrophage superoxide production following stimulation with sIC in two macrophage cell sources. A) Superoxide production increases with prolonged *in vitro* stimulation of RAW264.7 with PEG precipitated soluble immune complexes (sIC). RAW264.7 were plated at a density of 100,000/well in a 96 well black plate and incubated at 37° C, 5% CO_2 overnight. Then, either 1X PBS (negative control) or sIC were applied and incubated for 72 hours at 37° C, 5% CO_2. The media was then removed and fresh media containing 25 µM DHE was applied for a 30-minute 37° C, 5% CO_2 incubation. The graph displays superoxide production from *in vitro* RAW264.7 stimulation with sIC isolated by PEG precipitation from three different adult horses and is representative of two separate experiments. The error bars represent SEM and $p < 0.0001$. B) Equine primary PAM were harvested via BALF, washed and isolated. The primary equine PAM were stimulated for 72 hours with PEG-precipitated sIC from donor horse sera (Corning, Corning, NY). Briefly, a 96-well plate was seeded with approximately 50,0000 PAM/well (mixed population) in duplicates. Then, either 1X PBS (negative control) or sIC (from DHS or synthetic sIC made from eq. IgG + ovalbumin) were applied and incubated for 72 hours at 37° C, 5% CO_2. Synthetic sIC were created by incubating 1:1 of DHS with ovalbumin at 37° C, 5% CO_2 for 12 hours. The media was then removed and fresh media containing 25 µM DHE was applied for a 30 minutes 37° C, 5% CO_2 incubation. In both A and B, recombinant equine interferon gamma (10 ng/mL; R&D Systems) was applied simultaneously as the sIC stimulation to upregulate the FcγR. ROS were measured via RFU using an OMEGA plate reader at 544/620nm excitation/emission, respectively. Trends were similar although there was no statistical significance.

Conflict of Interest

The authors report no conflict of interest.

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CHAPTER 5. GENERAL CONCLUSIONS

The development of the immune system is a complex, dynamic, and ever-adapting network of cellular communication that changes throughout the life of an organism. Orchestration between genetic programming, nutrition and a plastic response to ever-changing environmental antigens all influence neonatal immune development. Early neonatal development of the immune system is a multifaceted subject and varies not only according to age but species as well. Largely, across all species, the neonatal immune system renders them more susceptible to disease, particularly pneumonia, when compared to the healthy adult [1].

Specifically, within the adult horse, the immune system is able to mount an effective Th1- or pro-inflammatory immune response against a pathogen like *R. equi* effectively clearing the organism via a robust phagocytic respiratory burst before it ever causes an infection within the host. Despite the fact that the field of equine neonatal immunology has made significant progress over recent years, gaps remain particularly with respect to site-specific immune responses to intracellular pathogens like *R. equi*. These gaps are most evident relating to site-specific pulmonary cytokine and cellular changes that occur within the first year of a foal's life. How these changes impact the principal cell that succumbs to infection with *R. equi*, the macrophage, remain yet to be determined.

Furthermore, controversy exists as to whether foals are Th2-, regulatory-, or anti-inflammatory- biased rendering them more susceptible to pneumonia due to an inability to mount an appropriate Th1- or pro-inflammatory response. Insofar as these neonatal regulatory or anti-inflammatory-biased responses do not result in classically activated

macrophages that possess the ability for increased intracellular killing of macrophage-tropic organisms through effective production of reactive oxygen and nitrogen species. The problem remains that, to date, no studies have investigated the pulmonary, site-specific innate immune environment signals that initially established this immune bias. Previous equine studies removed PBMC from young foals and stimulated them *ex vivo* which demonstrated that foals were a virtually in capable of producing IL-4 [2]. Our studies were performed using *in vivo* stimulation of heat-kill *R. equi* followed by analysis of the cytokine profile within the BALF.

The first two investigations contained in this dissertation sought to uncover the implicit bias of neonatal foal pulmonary alveolar macrophages by investigating their surrounding pulmonary cytokine milieu as well as how a pulmonary alveolar macrophage removed from that environment functioned through *ex vivo* bacterial infection assays. Furthermore, how the cytokine milieu as well as how macrophage phenotype and function changed over the first year of life were investigated.

In the last investigation of this dissertation, age-related changes regarding circulating soluble immune complex concentration were investigated as an additional immune differential affecting macrophage phenotype within the neonatal foal. Moreover, a potential mechanism of action behind the observed success, exemplified by a decrease in the incidence and severity of rhodococcal pneumonia in foals through prophylactic treatment with hyperimmune plasma, was offered.

PAM from Foals are Biased Toward a Regulatory/Tissue Repair Phenotype

The BALF from a cohort of foals was measured via ELISA for IL-4 and TNF-α concentrations at the following weeks of life: 1, 4, 8, 26, and 52. In foals less than six

months of age, IL-4 concentrations were observed to be increased compared to later in life while TNF-α concentrations remained consistent over the first year of life in the foal. IL-4 is the chief cytokine that induces a regulatory or tissue repair phenotype of the macrophage and these data provide evidence contributing to the understanding of why foal PAM are less efficient killers of intracellular pathogens. These data also reveal a potential reason explaining why foals are more susceptible to pneumonia caused by *R. equi* when compared to adults.

Decreased Capacity for Foal Macrophages to Produce Reactive Intermediates

The PAM and PBMC from the same cohort of foals were isolated from the BALF and blood, respectively, and were analyzed, *ex vivo*, for production of reactive intermediates and cytokine expression and then compared to the cells similarly derived and tested from adult horses. Fluorimetry was used in order to measure the production of superoxide, via DHE, and peroxynitrite, via DHR, in foal and adult macrophages following *in vitro* infection with *R. equi*, strain T194 transformed with either dsRed or GFP. Bacterial load was measured using fluorimetry. The results demonstrated that foal PAM and MDM contained significantly higher intracellular loads of *R. equi* while simultaneously producing less reactive intermediates when compared to the adult PAM and MDM. These results indicate that foal PAM and MDM removed from their immediate environment have a dampened microbicidal function compared to the adult.

To investigate an extrinsic mechanism behind macrophage activation, foal and adult pulmonary alveolar macrophages and monocyte derived macrophages were stimulated with CHO cells expressing ectopic equine CD154 [3], a receptor present on lymphocytes that functions to activate antigen-presenting cells following its binding to

CD40. Following stimulation, reactive intermediate production in foal macrophages was of significantly less magnitude than reactive intermediate production from adult macrophages exogenously stimulated in the same manner. Ultimately, these data support the hypothesis that PAM from foals are biased toward a regulatory- or tissue repair-phenotype.

Decreased Circulating sIC in Foals Compared to Adults

To investigate another potential inciting cause behind the inherent bias of the regulatory and tissue repair macrophage phenotype observed in foals, concentrations of soluble immune complexes were measured in neonatal foals and compared to corresponding concentrations in adults. Soluble immune complexes activate macrophages by binding to Fc gamma receptors (FcγR) present on the surface of macrophages [4]. Previous work has demonstrated an alternative mechanism for intracellular killing of *Leishmania amazonensis* through macrophage activation via cross-linking of immune complexes bound to FcγR [5]. Furthermore, given the reported success of the prophylactic use of equine hyperimmune plasma in reducing the severity and incidence of pneumonia caused by *R. equi* [6-10], a potential hypothesis was offered regarding soluble immune complex activation as a prospective mechanism.

Blood was drawn from neonatal foals and adults and sIC were isolated using a PEG6000 precipitation method previously published [11]. The sIC concentration of neonatal foals 12 hours following colostrum ingestion was significantly lower than when compared to the adult concentrations despite no significant difference between the immunoglobulin concentration. Additionally, foals had significantly less protein content within their measured sIC than compared to the adult although this is less impactful given

the lower normal concentration range of sera protein from neonatal foals. Collectively, these data support the hypothesis that foals have less circulating sIC than adult horses.

Future Directions

Several potential reasons why foals have an increased susceptibility to pneumonia caused by *R. equi* were uncovered within these investigations; however, the full picture remains elusive. Site-specific baseline cytokine populations need to be characterized overtime to reveal other important changes. Of note, IFN-γ, IL-10, IL-12, IL-13, IL-1β baseline changes within the BALF over the first year of life would be particularly interesting to characterize to uncover a greater picture of the true milieu. In concert with the change in cellular population, chemokines specific to the changing cell population would increase understanding of the spatiotemporal control and aid in determining the phenotypic changes of all cellular populations, especially the lymphocytes, both within the BALF as well as the systemic circulation. This information would aid in determining if BALF lymphocytes were also virtually incapable of Th2 responses or tissue repair- and anti-inflammatory responses similar to PBMC responses previously observed [2].

Aside from increasing the knowledge regarding a more complete cytokine milieu profile as well as a complete phenotypic cellular characterization across time, determining the source, particularly of IL-4, would increase knowledge of innate immunity uncovering potential new mechanisms behind neonatal immune responses to pathogens. Determining the cellular source of the characterized cytokines may be investigated through mRNA expression or *in situ* hybridization of tissue collected from

aging foals. This could potentially reveal immune functions of cells previously not regarded within innate immune realm.

Regarding the macrophage function, despite the various stimulants and *in vitro* mechanisms employed, the functionality of the foal macrophage could not be overcome via exogenous activation (equine reIFN-γ, TLR activation (heat killed *R. equi*), CD154, and sIC). This suggests that the external environment may not be all that is implicated regarding reduced functionality within foal macrophages. Therefore, determining which extrinsic factors are needed for macrophage function as well as which inherent macrophage mechanisms, or lack thereof, are responsible for this reduced functionality is vital. Investigating a potential combination of extrinsic factors working in concert with inherent macrophage mechanisms may reveal a reason for the observed decreased capacity for reactive intermediate production. Specifically, regarding external factors, recent attention has been given to IL-25, IL-33, and TSLP in priming type 2 immunity and activating innate lymphoid cell 2 (ILC2) [12, 18].

Intrinsically related to the macrophage, studying metabolic shifts regarding macrophage activation could provide a potential new area to study regarding these age-related changes as well as how these cells function on a physiologic basis [13]. Additionally, characterization of the downstream signaling pathways juxtaposing adult from neonatal macrophages could uncover a novel avenue for alternative treatments to address epigenetic changes that alter immune function in neonates not only in macrophages but lymphocytes as well.

Concerning lymphocytes, recent studies have demonstrated that T cell epigenetic changes such as differential methylation of the Th2 locus are different between neonates

and adults making neonatal immune responses primed toward Th2 cytokine production and subsequent development of Th2 effector cells [14]. Interesting, Ribeiro-do-Couto *et al.* reported that naïve Th cells recovered from the umbilical cord produced significant amounts of another Th2 cytokine, IL-13 [15]. Furthermore, Webster *et al.* discovered a potential reason for this including epigenetic changes related to differences in chromatin structure causing hypersensitivity, hypomethylation, and permissive histone modifications present in Th2 cells [16].

Furthermore, exploring the pulmonary milieu of receptor expression among immune cells is intriguing. Two types of IL-4 receptors exist. Type 1 exclusively binds IL-4 and is comprised of IL-4Rα and γc and type II, which binds both IL-4 and IL-13 and is comprised of IL-4Rα and IL-13α-1 subunits [17, 18]. Interestingly, non-hematopoietic cells express type II receptors while B and T cells express type 1 and macrophages express both types[18]. Evidence exists that downstream signaling events following IL-4 binding to the surface of macrophages depends on the presence of receptor expression, IL-4Rγc vs. IL-4Rα, and of cytokine availability[19, 20]. Determining a difference in receptor expression in foal antigen presenting cells vs. adult antigen presenting cells may provide additional reasons that explain the propensity of foal macrophages to adopt a regulatory or tissue repair phenotype.

Finally, a greater characterization of the antibody isotypes as well as the antigens contained within the circulating sIC population both *in vivo* from aging foals compared to adults and within hyperimmune plasma products is paramount. Although success has been observed, the use of HIP to prevent pneumonia in foals caused by *R. equi* remain controversial and the different proportions of the seven different isotypes present within

HIP vary greatly lot to lot and supplier to supplier [21]. This observed variation could be a reason explaining the variable success rates reported in Chapter 4. Furthermore, following identification of a consistent isolation, concentration ratio, and composition and characterization of sIC isolated from adults, sIC could be used to investigate macrophage activation and killing assays in order to determine an alternative mechanism for treatment of microbial infections.

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APPENDIX. AGE-RELATED VARIATION IN THE CELLULAR COMPOSITION OF EQUINE BRONCHOALVEOLAR LAVAGE FLUID

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Background: Previous reports reveal variation in the cellular composition of equine bronchoalveolar lavage fluid (BALF).

Objectives: The purpose of this study was to compare the profiles of BALF from horses to assess age-related differences. Serial BALF samples were collected from the same individuals over a one-year period to identify changes in individual animals as they aged.

Methods: Collection of BALF was performed on horses aged one week and one, 2, 6, and 12 months. Total nucleated cell count (TNCC), protein concentration, and cytology were assessed. Longitudinal analysis was performed and compared to healthy adults.

Results: Foals at one week and 6 months of age had significantly higher TNCC than adults (medians: 320/µL, 285/µL, and 90/µL, respectively); no differences in total protein were found. Foals at one month had the highest proportion of macrophages (median: 87.3%), differing significantly from both yearlings and adults (medians: 45.5% and 48.7%, respectively). Foals aged one week and one month had significantly lower proportions of lymphocytes than yearlings and adults (medians: 3.2% and 4.7% vs 43.2% and 45.8%, respectively). Eosinophil percentage was lowest in foals aged one week, one month, and 2 months (median: 0.0%) and highest in foals aged 6 months (median: 2.2%).

Mast cell percentages were highest in yearlings and adults (medians: 2.2% and 3.3%, respectively) and neutrophil percentage was highest in foals aged one week (13.7%). **Conclusions:** Cytologic profiles of BALF from foals and adult horses differed considerably. Significant changes in TNCC and percentages of lymphocytes, macrophages, and eosinophils occurred with age.

Introduction

Bronchoalveolar lavage (BAL) is frequently performed as part of a diagnostic workup for equine respiratory disease. Certain conditions are associated with altered cellular composition of bronchoalveolar lavage fluid (BALF). Diseases causing impaired lower airway function in which BALF analysis is helpful in achieving a diagnosis and/or monitoring a response to therapy include, but are not limited to, recurrent airway obstruction, silicosis, exercise-induced pulmonary hemorrhage (EIPH), and inflammatory airway disease (IAD) [1–3]. Bronchoalveolar lavage is most commonly performed in mature horses; however, it is occasionally performed in foals for both research and clinical intentions. Respiratory disease is a major cause of morbidity and mortality in foals, and BAL may be utilized in foals with clinical pneumonia that is refractory to treatment [4–6]. The cellular composition of BALF collected from horses with altered lower airway function is often characteristic for each disease entity; however, age-related differences in BALF have not been well characterized in a cohort of healthy horses.

Age-related changes in the cellular composition of BALF have been well documented in human beings. For example, an increase in total lymphocytes occurs in adults with age, and younger children are reported to have higher percentages of granulocytes than older children [7, 8]. An age-associated increase in the percentage of

lymphocytes in BALF collected from healthy adult Beagles was recently reported [9]. In the horse, immunophenotypic analysis of cells recovered from BALF revealed a decreasing CD4-to-CD8 lymphocyte ratio with age, as well as a higher proportion of lymphocytes in BALF from adult horses, supporting the notion that there is an age-related difference in the resident cell population of equine airways as well [10]. Recently, it was reported that healthy geriatric horses had an increased percentage of lymphocytes and a decreased percentage of macrophages in BALF when compared with young, healthy, and adult horses; similar age-dependent changes in mononuclear cell populations were identified in young foals [11,12]. In contrast, another study comparing airway inflammation in young adult vs. older sport horses found higher lymphocyte numbers in BALF collected from the younger age group [13]. These studies exemplify the need for age-specific data for proper cytologic evaluation of equine BALF.

In this study, we compared BALF cytology from healthy foals and adults with particular attention given to how resident cell populations evolve with age. To further characterize the effect of age on resident cell populations in the lung, BALF samples from the same cohort of foals were collected over a one-year period beginning at one week of age to identify changes in cell populations over time.

Materials and Methods

Animals

Inclusion criteria, prior to admission into the study, included a physical examination and a CBC, including leukocyte differential count and fibrinogen concentration within the respective RI. Subjects included 4 Quarter horse colts, one Quarter horse filly, 4 Thoroughbred colts, and one Thoroughbred filly. All study subjects

were housed in the same premise under similar conditions throughout the study. However, housing requirements varied slightly for each individual depending on birth date and age. In general, decisions regarding housing reflected the environmental conditions of the temperate climate. The subjects were housed in outdoor settings with a shelter available. These shelters were Iowa State University Institutional Animal Care and Use Committee (IACUC) approved and monitored with consideration to air quality. For this longitudinal study, samples were collected at defined time points according to age of the subject, beginning at approximately one week of age and extending to approximately one year of age. Specifically, BAL was performed on foals at the following ages (1–3 days): one week, one month, 2 months, 6 months, and 12 months. Adult samples were obtained from 4 healthy, adult geldings with no evidence of respiratory disease during exercise or at rest. The geldings were routinely exercised under saddle and on the lunge line and no exercise intolerance or evidence of respiratory disease (cough, nasal discharge, etc.) were noted. The geldings were 11, 13, 14, and 17 years of age and of the following breeds: Appendix Quarter horse, Warmblood/Thoroughbred cross, and 2 Thoroughbreds, respectively. The study was approved by the Iowa State University IACUC.

Bronchoalveolar Lavage Technique

Adult horses were sedated with intravenous detomidine (0.01 mg/kg) and butorphanol (0.01 mg/kg) prior to the procedure. A sterilized Olympus VQ-8303A (9 mm OD) videoendoscope was used to identify the airway of interest. From the left main stem bronchus, the scope was advanced caudo-dorsally parallel to the median plane in the standing, sedated adult until the scope was gently wedged in the selected airway.

Mepivicaine (2% solution, not more than 20 mL) was instilled during advancement of the endoscope to limit coughing and discomfort. Residual mepivicaine was immediately diluted with sterile saline used for the lavage. Lavages were performed using 480 mL of 0.9% sterile saline in 60 mL aliquots in the adult. Similarly, a sterilized pediatric videoendoscope (GIF-XP160 280 [5.9 mm OD]; Olympus, Center Valley, PA, USA) was used with 280 mL of sterile saline in 35 mL aliquots for obtaining BALs from heavily sedated foals (0.8 mg/kg xylazine IV; 5.0 mg butorphanol IV). In both foals and adults, the total volume of saline was utilized to perform the lavages. Samples from individuals were pooled and 1 mL aliquots were submitted for analysis.

BALF Analysis

Total protein was determined on BALF using a refractometer (TS Meter; American Optical, Southbridge, MA, USA). Total nucleated cell count (TNCC) was measured via laser flow cytometry using the ADVIA 120 hematology analyzer (Siemens Corporation, Washington, DC, USA). Cytocentrifugation of BALF was performed using a Shandon Cytospin3 (ThermoScientific, Waltham, MA, USA) 72g for 10 min (low acceleration). Air-dried slides were then stained with modified Wright's on an automated stainer (Siemens Hematek; Siemens Corporation). Determination of differential cell counts was done under 1000x magnification with oil immersion by counting 300 nucleated cells (epithelial cells excluded) by a board-certified clinical pathologist (SJH) without knowledge of animal age and time point of collection.

Datasets and Statistical Analysis

For analytic purpose, the samples were divided into 2 categories: a pooled dataset representing all samples collected and a second dataset representing BALF from foals for which samples were available at all time points throughout the study. The adult group (n = 4) represented a third group that was not part of the longitudinal study but was included to better characterize potential age-related differences. Normality tests (D'Agostino and Pearson tests) were performed on the datasets, and in some instances, the data were nonnormal; therefore, nonparametric tests were employed for all analyses. Differences between age groups (pooled samples) for TNCC and cell differential counts were assessed using Kruskal–Wallis one-way ANOVA on ranks and Dunn's test for multiple comparisons. Friedman test for repeated measurements followed by Dunn's multiple comparisons test were employed for the cohort group. Results were considered significant at $P < .05$. Statistical analysis was performed using NCSS10 Data Analysis (NCSS, LLC, Kaysville, Utah, USA) and GraphPad Prism (GraphPad Soft-ware, Inc., La Jolla, CA, USA) software.

Results

Study Population and BALF collection

Although 10 foals were included in the study, BALF samples were available at every time point from only 4 foals (cohort group, n = 4). Attrition of subjects occurred during the study at various time points due to a variety of reasons, including paddock accidents leading to euthanasia, illness unrelated to the study, and sale of yearlings. As a result of this attrition, the number of BALF samples analyzed in each pooled age group is as follows: n = 10 (one week), n = 9 (one month), n = 9 (2 months), n = 8 (6 months), and

n = 6 (12 months). Approximate retrieval volume of BALF volume was 90%. No health-related difficulties encountered during the study were attributable to BALF collection. BALF total protein and nucleated cell counts. Total protein concentrations were negligible in BALF samples, and no significant differences were detected in total protein content between age groups (data not shown). In contrast, TNCC differed significantly over time (P = .01, Kruskal–Wallis one-way ANOVA on ranks). Adult horses had the lowest TNCC (median: 90 cells/μL) compared with higher counts for foals at one week (median: 320/μL), one month (median: 230/μL), 2 months (median: 340/μL), 6 months (median: 285/μL), and one year (median: 205/μL). Pairwise comparisons between age groups revealed that the BALF collected from foals aged one week and 6 months had significantly higher TNCCs than BALF collected from adult horses (P < .05, Figure A-1).

BALF cytology

The most profound changes in inflammatory cell percentages between age groups were found in mononuclear cells. There was a significant difference in the proportion of macrophages within BALF between age groups (pooled data, P < .005, Kruskal–Wallis one-way ANOVA on ranks, Figure A-2A). Foals at one month of age had the highest proportion of macrophages (median: 87.3%), followed by foals at one week of age (median: 81.6%), foals at 2 months of age (median: 66.0%), and foals at 6 months of age (median: 60.5%). In contrast, BALF collected from yearlings and adult horses had lower proportions of macrophages (medians: 45.5%, and 48.7%, respectively). Pairwise comparisons between age groups showed significant differences in macrophage percentages between one- month-old foals compared with both yearlings and adults (P <

.01 and P < .05, respectively). Lymphocyte percentages within BALF also differed significantly between age groups (pooled data, P < .0001, Kruskal–Wallis one-way ANOVA on ranks, Figure A-2B). Foals had a lower proportion of lymphocytes at age one week (median: 3.2%), one month (median: 4.7%), 2 months (median: 10.7%), and 6 months (median: 28.0%) of age compared to adults (median: 45.8%). The proportion of lymphocytes in yearlings was similar to adults (median: 43.2%). Pairwise comparisons between age groups detected significant variation between the following age groups: one-week-old foals vs 6-month-old foals, yearlings, and adult horses (P < .01, P < .0005, and P < .001, respectively, Figure A-2B), one-month-old foals vs yearlings and adult horses (P < .01, Figure A-2B).

Neutrophil percentages were highest in foals aged one week (median: 13.7%) and lowest in adults (median: 2.7%; Figure A-2C); however, this difference was not statistically significant (P = .158). The proportion of neutrophils at the other time points sampled was as follows: one-month median: 5.0%, 2-month median: 4.7%, 6-month median: 5.2%, and 12-month median: 4.0%, Figure A-3C. There was a significant difference in eosinophil proportions between age groups overall (P < .005, Kruskal–Wallis one-way ANOVA on ranks, Figure A-2D). The percentage of eosinophils in BALF samples was lowest in foals at the first 3 time points sampled (median: 0.0% at 1 week, 1 month, and 2 months) and highest in 6-month-old foals (median: 2.2%, Figure A-3D). Yearlings had a higher proportion of eosinophils than adults (medians 1.5% and 0.3%, respectively). Significant differences in eosinophil percentages between specific groups were found between 6-month-old foals and foals aged one week and one month (pairwise comparison, P < .01 and P < .05, respectively). Lastly, the percentage of mast

cells in BALF tended to increase with age (Figure A-3E). Foals aged one month and 2 months had the lowest percentage of mast cells (median: 0.0%), followed by foals aged one week and 6 months (median: 0.3%). A higher proportion of mast cells were found in yearlings (median: 2.2%) and adult horses (median: 3.3%). Although the overall Kruskal–Wallis test for mast cell percentages had a $P < .05$, pairwise comparisons between groups failed to identify significant differences. BALF cytology within the same cohort of foals over time. Bronchoalveolar lavage fluid samples collected from the same 4 horses over a one-year period exhibited similar temporal changes in the cell proportions (Figure A-3). The proportion of lymphocytes in BALF significantly increased with age within the same cohort of individuals ($P = .0005$, Friedman test). Pair-wise comparison between groups showed a significant difference in lymphocyte percentage in these animals at age one week and 12 months (median: 2.5% vs 37.7%, respectively, $P < .05$, Figure A-3A). Similarly, the percentage of macrophages significantly decreased in BALF with age within the same cohort of foals ($P < .005$). Significant differences between the foals at age one month and 12 months were identified via pairwise comparison (median: 90.0% vs 53.7%, respectively, Figure A-3A). The tendency for decreasing neutrophil percentage, as well as increasing eosinophil and mast cell percentage with age was likewise noted within the cohort group (Figure A-3B). The increase in eosinophil percentage over time within the cohort group was significant ($P < .05$); however, pairwise comparisons failed to identify significant differences at specific ages.

Representative images of BALF from the same horse at various ages are provided for comparison (Figure A-4).

Discussion

This study compared the cellular composition, protein content, and TNCC for BALF collected from healthy foals of various ages and adult horses. Additionally, this is the first study to assess age-related changes within the same cohort of foals over a one-year period. Our results indicate significant differences in the proportion of both lymphocytes and macrophages present in BALF between foals and adult horses. The percentages of both eosinophils and mast cells also varied between age groups: eosinophil percentage was highest in the 6-month-old group, while mast cell percentages tended to increase with age. These differences emphasize the importance of considering age in the interpretation of BALF in the horse.

In healthy adult horses, macrophages are typically the most common cell type recovered in BALF [14,15]. Macrophages are present at all levels of the respiratory tract but are particularly numerous in the terminal bronchioles and alveoli [16]. Macrophages are a major component of innate immunity, serving a multitude of functions including phagocytosis and killing of microbial pathogens, production of cytokines, and antigen presentation to lymphocytes [17]. Pulmonary alveolar macrophages (PAM) are the principal or only phagocyte tasked with clearance of foreign particles and microbial pathogens from the terminal airways and alveoli, particularly as the mucociliary apparatus serves the more proximal airways. Within the distal airways and alveoli, macrophages can migrate away to become entrapped in the mucociliary apparatus, effectively acting as a shuttle mechanism, or can migrate to lymphatic or blood vessels for removal from the terminal airways [18]. Pulmonary alveolar macrophages also serve as antigen-presenting cells to initiate cell-mediated and humoral defenses; however, they are less effective at direct stimulation of T cells than resident macrophages in other

organs [19] The BALF cellular profiles from young foals in our study had a substantially higher percentage of macrophages (up to 1.89 higher) than adult horses, and the percentage of macrophages decreased with age. In young animals, innate immunity may represent a more significant component of their pulmonary immune defense [20, 21]. To our knowledge, there are few studies describing an increased proportion of macrophages in BALF in the young of other species; however, this finding has been described previously in very young children. Children in the youngest age group sampled (< 2 years) had a significantly increased proportion of macrophages compared to children in the older age groups (2–5 years and 6–17 years). In addition, the PAM collected from children within the youngest age group had decreased function [22] The trend for a decreasing proportion of macrophages in BALF continues as people age, as elderly people are reported to have a lower percentage of macrophages than younger adults [21] Macrophages isolated from BALF of older adults released more superoxide in response to stimulation than macrophages from younger adults [23]. In swine, PAM isolated from younger pigs likewise showed decreased oxidative burst in response to stimuli than PAM from older pigs [24]. Additional studies are needed to determine if PAM function changes with age in the horse.

Our data support the findings of previous studies suggesting that lymphocyte percentages in BALF increase with age in the horse [10–12, 25]. The relative increase in lymphocytes in BALF seen in adult horses compared with foals could reflect an increase in memory cells associated with increased antigen exposure. To our knowledge, increased lymphocyte percentages in BALF are not currently associated with any specific equine respiratory diseases. In human medicine, an increased percentage of lymphocytes

is considered evidence of inflammation in BALF, and has been associated with certain pathologic conditions such as bronchiolitis obliterans, *Mycobacterium abscessus* infection, and asbestos exposure [26–29]. It is therefore conceivable that as the use of BAL as a diagnostic tool in veterinary medicine evolves, an association between alterations in BALF lymphocyte proportions and specific respiratory conditions may be identified.

Tendencies for a decreasing percentage of neutrophils in BALF concurrent with increasing percentages of mast cells and eosinophils were identified in the cohort population that was studied over one year, as well as in the pooled samples at those time points. It may be worthwhile to consider the potential for age-related changes when interpreting BALF cytology, as most inflammatory conditions of young adult and aged horses are characterized, at least in part, by an increase in neutrophils, mast cells, and/or eosinophils. For example, an increase in the percentage of one or more of these cell types is used to help define horses suffering from IAD (> 10% neutrophils, > 5% mast cells, > 5% eosinophils—or any combination) [1, 30–33]. The youngest age group of foals sampled in this study (one week of age) had, on average, a higher proportion of neutrophils than the other age groups; however, this finding was not statistically significant. Previous studies comparing BALF cytology between mature and immature subjects have likewise identified differences in neutrophil counts. For example, studies in both human beings and dogs have identified an increased percentage of neutrophils in young animals compared with adults in health [7,9]. In an experimental mouse model, younger animals had a more pronounced inflammatory airway response to antigen than adult animals [34]. As neutrophils represent the first line of defense against pathogens

and are a primary effector cell of innate immunity, this increase could represent a normal physiologic response to inhalation of particulate matter after birth. Alternatively, some of the foals included within our study could have suffered from subclinical respiratory disease during the neonatal period. The neutrophil percentage decreased by the next time point (one month of age) to levels comparable to the adults. The clinically healthy foals in this study also exhibited an increase in both eosinophils and mast cells by 6 months of age. This increase could be caused by a variety of factors, such as maturation of pulmonary innate defense mechanisms, a normal response to typical housing conditions, chronic exposure to respirable particles, or some other effect that has not been definitively established. Recently, a potential link between inhalable particulates and eosinophilic inflammation in BALF in Thoroughbreds entering training was established [35].

Regardless of cause, our study indicates a proclivity of healthy weanlings and yearlings to have modest increases in percentages of mast cells and eosinophils in BALF, and emphasizes the importance of interpreting changes in BALF cytology in the context of existing clinical signs. The difference in TNCC between immature horses and adults was somewhat unexpected. Although the approach to collection technique was similar, the endoscope used for BALF collection differed between young foals and adults. Nonetheless, the finding of higher TNCC in immature animals was still observed in 6-month-old horses and in yearlings, where the larger endoscope was utilized to perform the lavages. In the horse, higher TNCC counts were identified in young adult horses compared to an older age group in a prior study [13]. Consideration of an age-dependent effect on TNCC could be clinically relevant, as an elevated TNCC in BALF can be seen

with some respiratory diseases. Specific inflammatory conditions associated with an increased TNCC in equine BALF include IAD and EIPH [1, 2, 36, 37]. Our finding that age can be associated with significant differences in TNCC in BALF from healthy horses could be of particular significance in the context of diagnosing IAD, a disease affecting horses of all ages, particularly young horses. The BALF cytologic profile in horses with IAD is characterized by an increased TNCC as well as increased neutrophils, eosinophils, and/or mast cells [1]. Elevations in TNCC, however, are not specific for inflammation within the lower airways. For example, higher TNCCs have previously been reported in children compared with adults [7]. In the horse, higher TNCC counts were identified in young adult horses compared to an older age group in a prior study [13]. Additionally, Thoroughbred colts in training have been shown to have higher BALF TNCC counts, as well as an increased percentage of neutrophils and eosinophils, when compared with stabled colts of similar age [38,39]. In this context, it is important for clinicians and clinical pathologists to recognize that although an increase in TNCC can occur with airway inflammation in horses, it is not specific for inflammation and should be interpreted in conjunction with the signalment, history, and clinical signs. In conclusion, this study identified a progressive increase in the proportion of lymphocytes and a corresponding decrease in macrophages in BALF in horses with age. Additionally, immature horses had higher TNCC in BALF than the adults sampled. The cause for this age-related difference is unknown, and warrants further investigation. These findings exemplify the potential importance of age as a possible variable in the interpretation of equine BALF. As the clinical utility of BALF evaluation becomes more widely explored in younger horses, a larger study to establish age-specific RIs may be useful.

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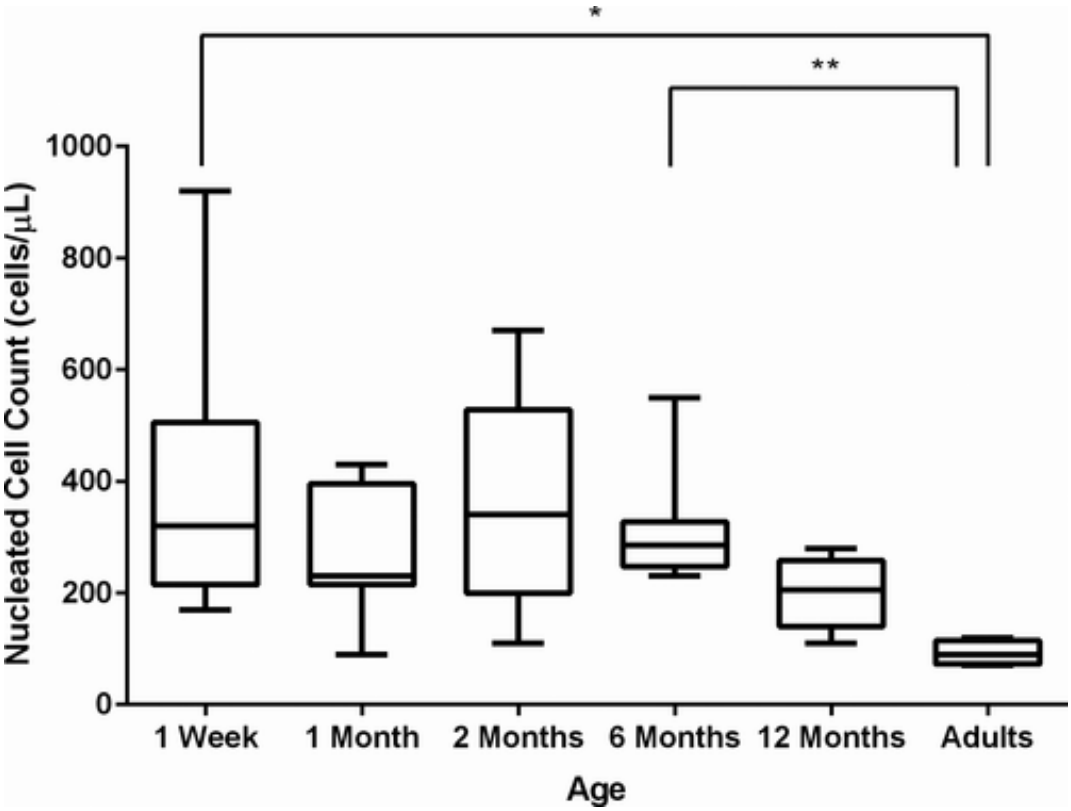

Figure A-1. Box plot of total nucleated cell counts (TNCC) per microliters in bronchoalveolar lavage fluid (BALF) from horses at various ages. Foals at one week and 6 months of age had significantly higher TNCC than adult horses. Data are expressed as median (horizontal line), interquartile range (box), and minimum/maximum value (whiskers; Kruskal–Wallis one-way ANOVA on ranks and Dunn's test for multiple comparisons; *$P < .02$; **$P < .05$).

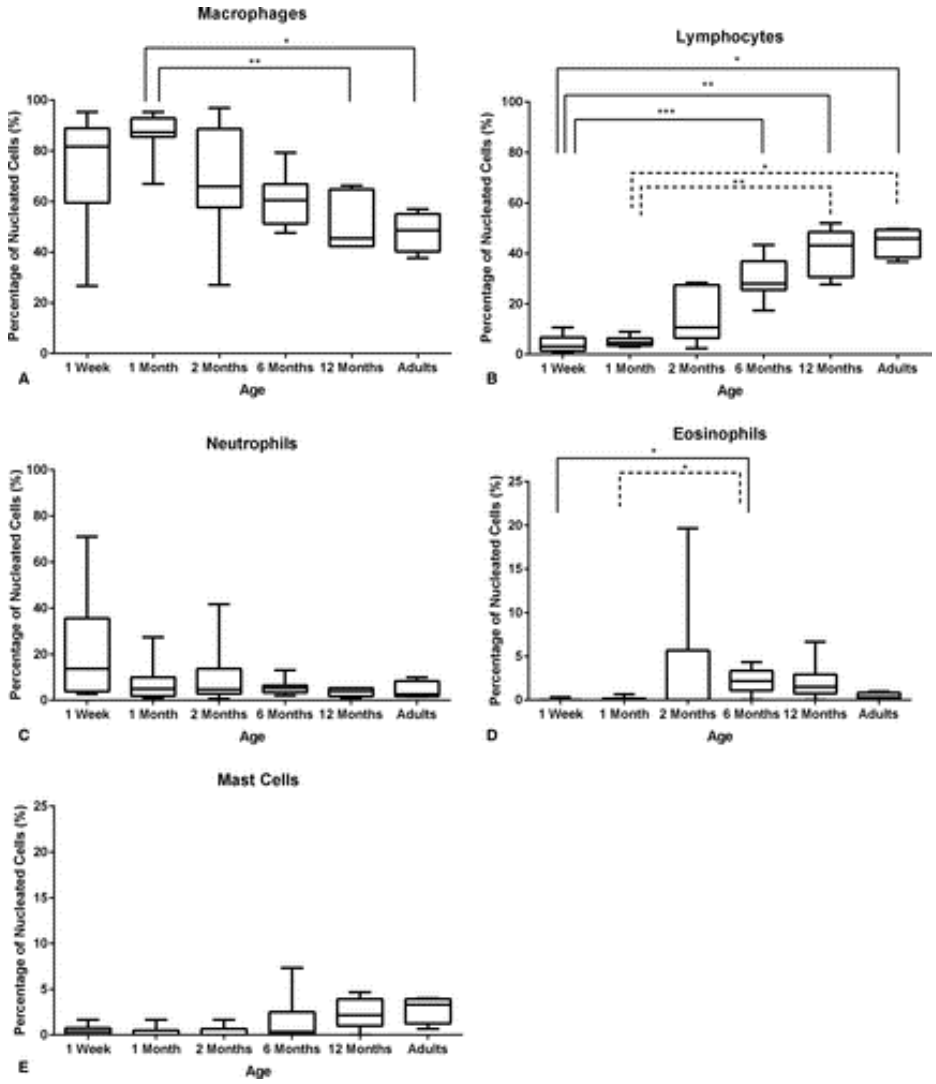

Figure A-2. Temporal changes in percentages of macrophages (A), lymphocytes (B), neutrophils (C), eosinophils (D), and mast cells (E) in bronchoalveolar lavage fluid (BALF) samples taken from horses at various ages. Horses at one month of age had a significantly higher percentage of macrophages than 12-month-old horses and adults (A; *P < .05; **P < .01). Lymphocyte percentage was lowest in horses aged one week, and differed significantly from adult horses as well as 12- and 6-month-old horses (B; solid line; *P < .001; **P < .0005; ***P < .01, respectively). Horses aged one month also had a significantly lower percentage of lymphocytes than adults and horses aged 12 months (B; dashed line; *P < .01; **P < .01). Foals at one week of age had a higher median percentage of neutrophils than other age groups; however, this finding was not statistically significant (C). Horses at 6 months of age had a significantly higher percentage of eosinophils than horses aged one week and one month (D; solid line, P < .01; dashed line, P < .05, respectively). Mast cell percentage tended to increase with age, with adult horses having the highest median percentage (E). Data are expressed as median (horizontal line), interquartile range (box), and minimum/maximum value (whiskers; Kruskal–Wallis one-way ANOVA on ranks and Dunn's test for multiple comparisons).

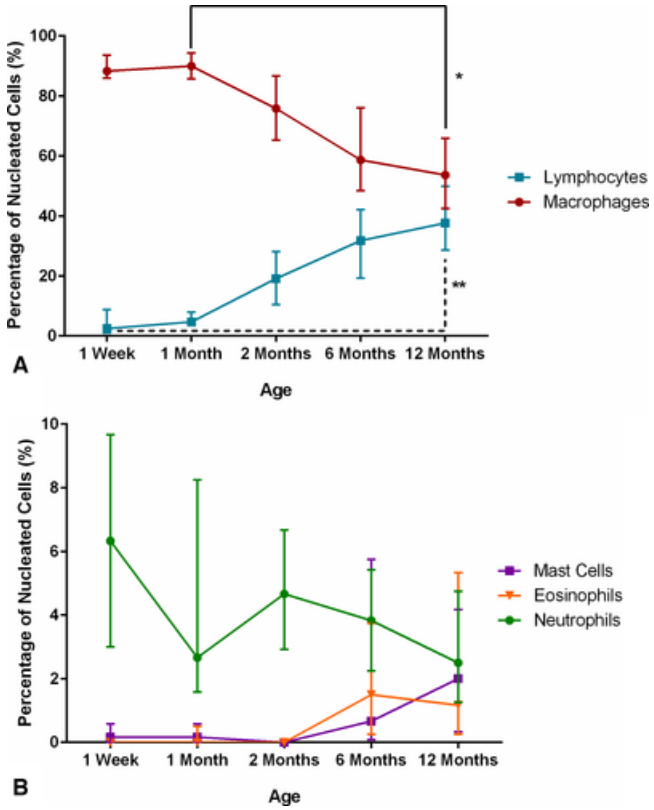

Figure A-3. Comparison of resident cell populations in bronchoalveolar lavage fluid collected from the same cohort of foals over a one-year period. Temporal changes were most profound in mononuclear cell populations (**A**). The foals had significantly higher percentages of macrophages at one month compared with 12 months of age (**P* < .05); lymphocyte percentages increased significantly over time (one week vs 12 months, ***P* < .05). Trends for decreasing neutrophils and increasing mast cells and eosinophils also were noted (**B**); however, these changes were not statistically significant within the cohort (*n* = 4, data expressed as median and interquartile range. One-way repeated measures ANOVA on ranks (Friedman Test) with Dunn's test for multiple comparisons).

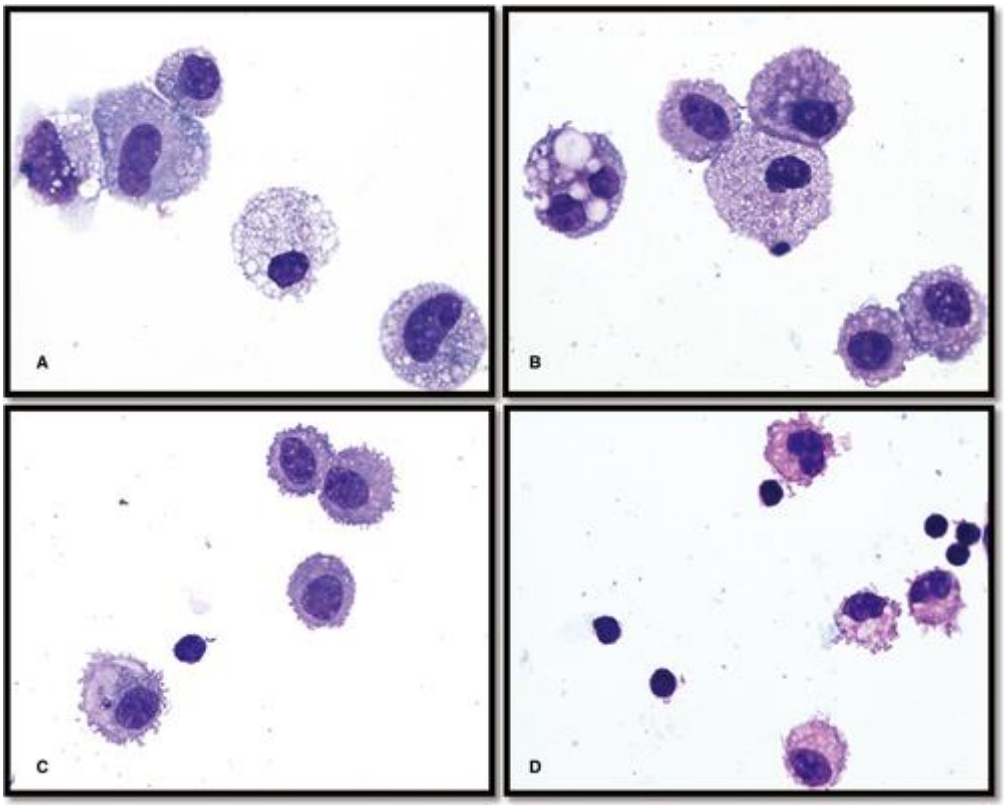

Figure A-4. Representative photomicrographs of cytocentrifuge preparations of bronchoalveolar lavage fluid (BALF) sampled from the same horse at various ages, modified Wright's, ×100 objective. (A) one week, (B) one month, (C) 6 months, and (D) 12 months. Note the greater proportion of macrophages relative to lymphocytes in the BALF sampled at ages one week, one month, and 6 months (A, B, and C) when compared to age 12 months (D).

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